Towards Development of Imidazolinone Herbicide Resistant Borage (*Borago officinalis*)

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ABSTRACT

Borage (*Borago officinalis*) is an annual herb plant for culinary and medicinal uses. Due to a high level of gamma-linolenic acid (GLA) in its seed oil and the health-related benefits of GLA, borage is commercially cultivated. However, a herbicide-resistant variety has not yet been developed for effective weed management in borage farming. Thus, this thesis aimed to create, identify and characterize ethyl methanesulfonate (EMS) induced borage mutants for herbicide imidazolinone resistance. An EMS-mutagenized borage population was generated by using a series of concentrations of EMS to treat M1 seeds. After screening M2 borage plants with the herbicide, tolerant plants were selected, self-pollinated and grown to their maturity. The offsprings were subjected to herbicide screening again to confirm the phenotype, resulting in identification of two genetically stable imidazolinone-resistant lines. Two acetohydroxyacid synthase (AHAS) genes, *AHAS1* and *AHAS2*, involved in the imidazolinone resistance were isolated and sequenced from both mutant (resistant) and wild type (susceptible) borage plants. Comparison of these *AHAS* sequences revealed that a single nucleotide substitution occurred in the *AHAS1* resulting in an amino acid change from serine (S) in the susceptible plant to asparagine (N) in the first resistant line. The similar substitution was later found in the *AHAS2* of the second resistant line. A KASP marker was developed for the *AHAS1* mutation to differentiate the homozygous susceptible, homozygous and heterozygous resistant borage plants for the breeding purpose. The *in vitro* assay showed homozygous resistant borage containing the *AHAS1* mutation could retain significantly higher AHAS activity than susceptible borage across different imazamox concentrations. The herbicide dose response test showed that the resistant line with the *AHAS1* mutation was tolerant to four times the field applied concentration of the “Solo” herbicide.

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LIST OF ABBREVIATIONS

AHAS: acetohydroxyacid synthase

ALS: acetolactate synthase

BLAST: basic local alignment search tool

COX: cyclooxygenases

CTAB: cetrimonium bromide

DGLA: dihomogamma linolenic acid

DTT: dithiothreitol

EMS: ethyl methanesulfonate

FAD: flavin adenine dinucleotide

FRET: fluorescence resonance energy transfer

GLA: gamma-linolenic acid

KASP: kompetitive allele specific PCR

LA: linoleic acid

LOX: lipoxygenases

PAs: pyrrolizidine alkaloids

PCR: polymerase chain reaction

PDC: pyruvate decarboxylase

PVP: polyvinylpyrrolidone

PVPP: polyvinylpolypyrolidone

RACE-PCR: rapid amplification of cDNA ends PCR

RDA: recommended dietary allowance

SNP: single nucleotide polymorphism

ThDP: thiamine diphosphate

1.0 INTRODUCTION

Borage (*Borago officinalis* L.), an annual herb, originates from the Mediterranean region. Historically, borage is used for culinary and medicinal purposes (Gerard, 1994). In the recent decade, borage oil has gained great attention and interest from medical and nutritional research due to its high content of gamma-linolenic acid (GLA) (Huang *et al.*, 1995; Rahmatullah *et al.*, 1994*a, b*; Wolff and Sebedio, 1994). The oil containing GLA has shown positive effect in treating a number of clinical conditions caused by GLA deficiency in humans (Chapkin and Carmichael, 1990; Engler *et al.*, 1991; Engler *et al.*, 1992; Gibson *et al.*, 1992; Rahmatullah *et al.*, 1994*a, b*; Redden *et al.*, 1995). In addition, a study also suggests that consumption of borage oil can improve fatty acid metabolism and skin function in elderly people (Brosche and Platt, 2000). Because of these, many nutraceutical supplements, food products and body-care products have now been enriched with borage oil, resulting in a surge of the demand for borage farming.

The yield and quality of borage cultivation are determined by many factors, within which weed management is crucial. Weed is the major threat to crop production and it causes losses in billions of dollars (Basu, 2004). Due to lack of herbicide resistant variety, weeding for borage is still primarily relying on hand labors; however, weeding is constantly required and lasting until flowering when the plants are well established. Thus, weeding notably increases the cost of borage production, which leads to fewer farmers who are willing to cultivate borage in a massive scale. Consequently, the industry eagerly awaits development of a herbicide resistant borage cultivar (Wu, personal communication).

Among different groups of herbicides, imidazolinone herbicide controls a broader spectrum of weeds at low application rate. This group of herbicides inhibits the essential enzyme, acetohydroxyacid synthase (AHAS), which is also known as acetolactate synthase (ALS). AHAS

catalyzes the biosynthesis of branched chain amino acids in plants (Tan *et al.*, 2005). After the discovery of a variety of imidazolinone tolerant plant species with altered AHAS genes, many imidazolinone resistant crops have been developed through mutagenesis and selection utilizing conventional plant-breeding techniques (Al-Khatib *et al.*, 1998; Croughan, 1998; Newhouse *et al.*, 1991; Shaner *et al*., 1996; Tan *et al.*, 2005). Given the past achievement in the herbicide-resistant breeding in many crop species, imidazolinone resistant borage would also be possibly developed using a similar mutagenesis and screening strategy.

2.0 HYPOTHESIS AND OBJECTIVES

2.1 Hypothesis

Ethyl methanesulfonate (EMS) induces single nucleotide substitution of genes resulting in change of an amino acid in an enzyme. The chemical mutagenesis has been applied to produce imidazolinone-resistant crops by altering certain amino acids of AHAS, a target enzyme involved in the biosynthesis of essential branched amino acids. Therefore, the chemically induced mutagenesis of the herbicide-target enzyme could be also used to develop imidazolinone resistant borage.

2.2 Objectives

The objectives of this thesis research are as follows:

1) To generate an EMS-mutagenized population using a series of EMS solutions to treat borage seeds;

2) To screen the mutagenized population for imidazolinone resistance using a group 2 herbicide;

3) To elucidate molecular mechanisms underlying imidazolinone resistance by identifying mutations of AHAS genes in the mutant lines;

4) To carry out *in vitro* AHAS activity assay of imidazolinone resistant mutant plants;

5) To examine herbicide type and dosage response of imidazolinone resistant borage plants.

3.0 LITERATURE SURVEY

3.1 Borage

Borage, an annual herb from Boraginaceae family, is native to the Mediterranean region and has now naturalized worldwide. However, it is mainly grown commercially in the United Kingdom, the Netherlands, Canada, New Zealand and Poland, which accounts for 95% of global production (Kapoor and Nair, 2005). Borage is an erect and hispid plant that can normally grow up to 60 - 100 cm (2.0 - 3.3 ft) in height; and it has simple alternate leaves that are obovate, ovate or oblong with an obtuse apex. The stem is cylindrical, hollow and succulent. The stems, leaves and calyx are covered with stiff unicellular trichomes (Kapoor and Nair, 2005). Borage is also known as “star flower” because of the shape of the flower. The flowers vary in color including bright blue, violet, and pink, even white at different stages and between biotypes. Borage flowers are produced on scorpioid cymes which arise from the axils of the leaves at intervals on the stem. Flowering proceeds basipetally in the inflorescence and each inflorescence develops several flowers. Each flower contains a deeply 4-lobed ovary in gynobasic style. As the flower matures, it develops into 3 - 4 ovoid or oblong seeds. The seed coat will develop color from green to brown and then black signifying maturity. After then, the seed will abscise shortly. However, it should be mentioned that new varieties have been developed with the trait of “seed retention” (Kapoor and Nair, 2005; Montaner *et al.*, 2001; Wu, personal communication). Borage is an allogamous plant with an entomophilous pollination system; that means, insects such as bees take pollen grains and spread them onto neighboring flowers (Alvarez and Villa, 1992).

Borage has a long history of being used for food and medicine (Gerard, 1994). According to folklore, it was used as salad or vegetable by early Europeans. Also, it was considered to be heart-comforting and contain antidepressant qualities in medieval times. Borage was also believed to bring courage and joy (Gerard, 1994; Kapoor and Nair, 2005). Moreover, naturopathic practitioners use borage to treat many disorders. It can help to regulate metabolism and hormone secretion especially for female patients who suffer from premenstrual syndrome and menopause symptoms such as hot flash. Due to the anti-inflammatory and balsamic properties, borage is also a good remedy to alleviate and heal colds, cough, swelling of limbs and breathing problems (Gupta and Singh, 2010; Seif *et al.*, 2011). While mixed data indicates that borage oil is unlikely to have a major clinical effect on severe atopic dermatitis (skin disorder), it can still improve and maintain skin functions in subjects with dry, sensitive and aging skin conditions as well as mild atopic dermatitis (Brosche and Platt, 2000; De Spirt *et al.*, 2012; Foster *et al*., 2010; Kawamura *et al*., 2011).

According to USDA national nutrient database (2014), borage contains notable levels of vitamins, minerals and phyto-nutrients (Table 1). Every 100 g of fresh borage leaves can provide vitamin A and vitamin C at 140% and 60% of Recommended Dietary Allowance (RDA) respectively. It is also a good source of minerals especially iron, up to 41% RDA. Further, borage provides a good amount of micronutrients such as niacin at 26% of RDA from 100 g of fresh leaves.

Table 1. Nutritional values per 100 g of fresh borage leaves (USDA national nutrient database, 2014)

Principle	Nutrient Value	Percentage of RDA	Principle	Nutrient Value	Percentage of RDA
Energy	21 Kcal	1%	Vitamin A	4200 IU	140%
Carbohydrates	3.06 g	2%	Vitamin C	35 mg	60%
Protein	1.80 g	3%	Sodium	80 mg	5%
Total Fat	0.70 g	2%	Potassium	470 mg	10%
Cholesterol	0 mg	0%	Calcium	93 mg	9%
Folates	13 ug	3%	Copper	0.13 mg	15%
Niacin	0.90 mg	26%	Iron	3.30 mg	41%
Pantothenic acid	0.04 mg	1%	Magnesium	52 mg	13%
Pyridoxine	0.08 mg	7%	Manganese	0.35 mg	15%
Thiamin	0.06 mg	5%	Zinc	0.20 mg	2%

Borage is well known for rich GLA content (Figure 1) ranging from 16% to 28% in the seed oil (Table 2), and its total oil content in a seed can reach 27% to 37% (w/w) (Del Rio and De Haro, 1993; Kapoor and Nair, 2005). The variation in GLA content is contributed by multiple factors including geographical location, length of the light period during growing season, average temperature and diurnal temperature difference (Kapoor and Nair, 2005). Besides GLA, borage oil also contains a significant amount of linoleic acid (LA), one of essential omega-6 fatty acids, up to 38%, but there is no relationship between the content of LA and GLA. However oleic acid (18:1n-9) is reported to have an inverse relationship to GLA (Clough, 2001; Kapoor and Nair, 2005).

Figure 1. Chemical structure of gamma-linolenic acid (cis-6, cis-9, cis-12-octadecatrienoic acid) (Lipidmaps.org, 2014)

Table 2. Fatty acid composition of borage oil (adapted from Clough, 2001)

Fatty Acid	Common Name	Borage (% of the total fatty acid)
16:0	palmitic acid	10.2
18:0	stearic acid	3.3
18:1*n*-9	oleic acid	14.8
18:2*n*-6	linoleic acid	37.9
18:3*n*-6	gamma-linolenic acid	24.6
18:3*n*-3	alpha-linolenic acid	0.2
20:0	arachidic acid	0.2

Borage oil also contains minor components such as sterols, tocopherols and pigments. γ-tocopherols and δ-tocopherols can reach 650 ppm and 50 ppm respectively in the oil. The major proportion of sterols belongs to 4-desmethylsterol, whereas a small amount of 4-monomethylsterol and 4,4-dimethylsterol are present as well (Kapoor and Nair, 2005; Sensidoni *et al.*, 1995; Wretensjö and Karlberg, 2002). Just like other herb plants, pyrrolizidine alkaloids (PAs), a group of hepatotoxic compounds, can be found in borage leaves, flowers and seeds; however based on the available data, consumers need no concern to the toxicity from PAs

because the PA levels in borage oil is extremely far below the detection limit of conventional methods (Kapoor and Nair, 2005). Besides high oil content and rich GLA level, large seed size of borage also makes harvest and oil extraction much easier; thereby, borage is the most preferred source of GLA in comparison to other plants (Galwey and Shirlin, 1990).

3.2 Gamma-linolenic acid (GLA)

GLA is an 18-carbon polyunsaturated fatty acid containing three double bonds (Figure 1). It is an Δ-6-desaturated product in the metabalization of LA. However, the GLA formation can be restricted by many factors in the human body including nutritional deficiency, inflammatory conditions, certain diseases and life style (Figure 2). Also, arachidoinc acid and eicosapentaenoic acid in the body can inhibit the enzymatic activity of Δ-6-desaturase for GLA biosynthesis (Kapoor and Huang, 2006).

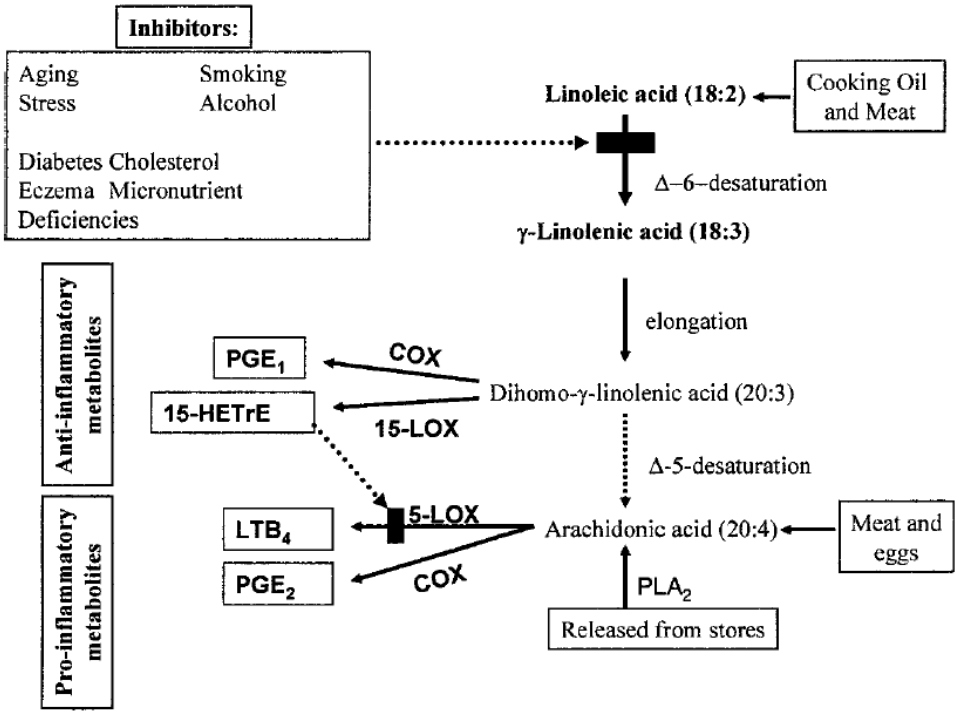


Figure 2. Biosynthesis of GLA as an intermediate compound in the metabolic pathway of LA.

COX, Cyclooxygenase; LOX, Lipoxygenase; PGE1, Prostaglandin E1; PGE2 , Prostaglandin E2; 15-HETrE, 15-hydroxy eicosatrienoic acid; LTB4, Leukotriene B4 (adopted from Kapoor and Nair, 2005).

The metabolic pathway of GLA in humans and animals has been reviewed (Kapoor and Nair, 2005) (Figure 2). After GLA is formed or administered, it is immediately elongated to dihomo-gamma linolenic acid (DGLA). DGLA is mostly acylated by acyl transferases to phospholipids moving to the cell membrane, but a small amount of DGLA is slowly converted to arachidoinc acid by the enzyme Δ-5-desaturase. DGLA competes with arachidoinc acid for enzymes COX and LOX to produce secondary messengers that are responsible for communications between cells and mediation of physiological effects. The COX products of DGLA include prostaglandins E1 and thromboxane A1, which are anti-inflammatory, vasodilatory and anti-aggregatory. By the action of enzyme 15-LOX, DGLA also produces 15-hydroxyl eicosatrienoic acid, which inhibits enzyme 5-LOX catalyzing arachidoinc acid to inflammatory compound, leukotriene B4, from inflammatory cells such as neutrophils (Figure 2) (Chilton *et al.*, 1996; Kapoor and Huang, 2006; Ziboh *et al.*, 2000). Plenty of research has proved the linkage between GLA and anti-inflammatory (Brzeski *et al.*, 1991; Calder and Zurier, 2001; Deluca *et al*., 1999; Gillis *et al*., 2004; Ziboh *et al*., 2004), and anti-cancer actions (Hrelia *et al.*, 1996; Kenny *et al.*, 2000; Jiang *et al.*, 1995; Mainou-Fowler *et al.*, 2001; Sagar *et al*., 1992; Watkins *et al*., 2005). Moreover, GLA also showed to be effective in treating diabetic neuropathy leading to improved blood flow and reduced tingling of extremities (Jamal, 1994; Kapoor and Huang, 2006). In addition, a number of studies investigating the role of GLA in cardiovascular health have suggested that dietary GLA reduces low-density lipoprotein cholesterol (Fukushima *et al.*, 1997), plasma triacylglycerols (Laidlaw and Holub, 2003), blood

pressure and smooth muscle proliferation (Fan *et al*., 1997). In light of the efficacy of GLA in treating physiological disorders and diseases caused by deficiencies in essential fatty acids and anti-inflammatory secondary messengers, several sources of GLA including borage oil have been developed (Horrobin, 1992; Kapoor and Huang, 2006; Wu and Meydani, 1996).

3.3 Imidazolinone herbicides in weed management

Not only to borage crops, are weeds ubiquitous threat to agriculture worldwide, costing farmers 20 billion dollars annually in US alone (Basu, 2004). Weeds are highly competitive wild plant species that persistently adapt in agricultural systems and lower productivity and quality of crop products (Yuan *et al*. 2006); therefore, weed management is an integral part of agriculture. Weed management involves the combination of preventative, physical, cultural, biological and chemical strategies (B.C. Ministry of Agriculture, 2012). However, most of them are time-consuming, labour-intensive and costly. As a result, the chemical strategy using herbicides has increasingly become a dominant weed control measure in recent decades (Wu, 2012; Yuan *et al*. 2006).

Herbicides represent a large array of chemical compounds able to kill weed plant species. They usually act at targeted sites of essential enzymes where metabolic function and energy transfer are taking place in plant cells, thereby inhibiting the enzymatic function (Cole *et al*. 2000; Duke, 1990). More than 60% of herbicides introduced in the last four decades are designed to interfere with the function of chloroplasts in plants, even though the action mechanism of some commercial herbicides is not yet fully clear (Wakabayashi and Boger, 2002). Based on modes of action, commercial herbicides are classified into 27 groups (Alberta Agriculture and Rural Development, 2014). Among them, the action mechanism of group 2 herbicides falls into the category of inhibition of amino acid biosynthesis; that is, these herbicides control weeds by

inhibiting the enzyme AHAS which is a critical enzyme in the biosynthesis of branched chain amino acids (leucine, isoleucine and valine) in plants (Duke, 1990). Group 2 herbicides consist of five chemical families including imidazolinones, sulfonylureas, triazolopyrimidines, pyrimidinylthiobenzoates and sulfonylamino-carbonyltriazolinones (Mallory-Smith and Retzinger, 2003). Imidazolinones include imazapyr, imazapic, imazethapyr, imazamox, imazamethabenz and imazaquin (Figure 3) (Tan *et al*., 2005), and all of them contain an imidazole moiety in the molecular structure (Vencill, 2002). Based on the second cyclic structure of the molecules excluding the imidazole ring, they can be further divided into three groups (Figure 3) (Tan *et al*., 2005).

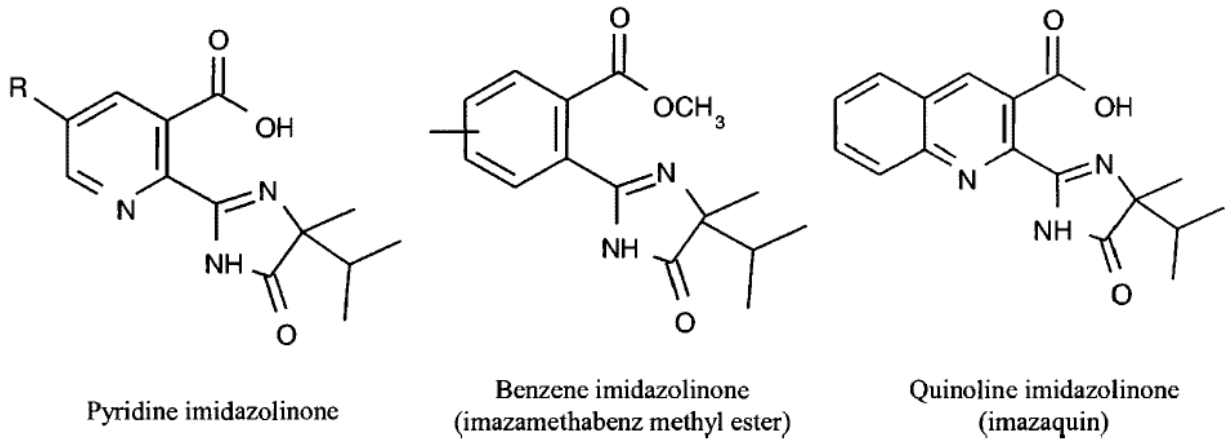


Figure 3. Imidazolinone herbicides; imazapyr: R = H, imazapic: R = CH_3, imazethapyr: R = $CH_3 - CH_2$, and imazamox: R = $CH_3 - O - CH_2$ (adopted from Tan *et al*., 2005).

Imazamethabenz contains a benzen ring, imazaquin has a quinoline moiety and the rest of imidazolinones are characterized by a pyridine ring. Four analogs within pyridine imidazolinones are differentiated by the R-group of the pyridine ring. Imazapyr, imazapic, imazethapyr and imazamox respectively have hydrogen (H), methyl (CH_3), ethyl ($CH_3 - CH_2$) and

methoxymethyl ($CH_3 - O - CH_2$) functional groups at R-position of the pyridine ring (Figure 3). Yet, the activity of AHAS inhibition among pyridine imidazolinones is very similar (Tan *et al.*, 2005). In fact, the functional groups are suggested to be related to certain characteristics of the metabolism of pyridine imidazolinones in plants (Tecle *et al.*, 1997). Besides the strong link between imidazole ring and AHAS inhibition, the second cyclic structures, pyridine, benzene and quinoline rings, also play important role in AHAS inhibition resulting in the different inhibition activities (Shaner and Singh, 1997; Tan *et al.*, 2005). Imidazolinone herbicides are effective to control a wide spectrum of grass and broadleaf weeds at a low application rate with low mammalian toxicity. Thus, imidazolinones possess many ideal traits for utilization in developing a herbicide resistant crop (Tan *et al.*, 2005).

3.4 Acetohydroxyacid synthase (AHAS)

Acetohydroxyacid synthase (AHAS) (EC 4.1.3.18), also known as acetolactate synthase (ALS), catalyzes the first reaction in the pathway for synthesis of the branched chain amino acids leucine, isoleucine and valine (Figure 4) in plants and many microorganisms (Duggleby and Pang, 2000). An unusual feature of the pathway is two parallel condensation reactions catalyzed by AHAS enzyme leading to the formation of valine and isoleucine (Duggleby and Pang, 2000): two pyruvate molecules produce carbon dioxide (CO_2) and 2-acetolactate - a precursor of valine and leucine; one pyruvate molecule and α-ketobutyrate form CO_2 and 2-acetohydroxybutyrate - a precursor of isoleucine (Duke, 1990).

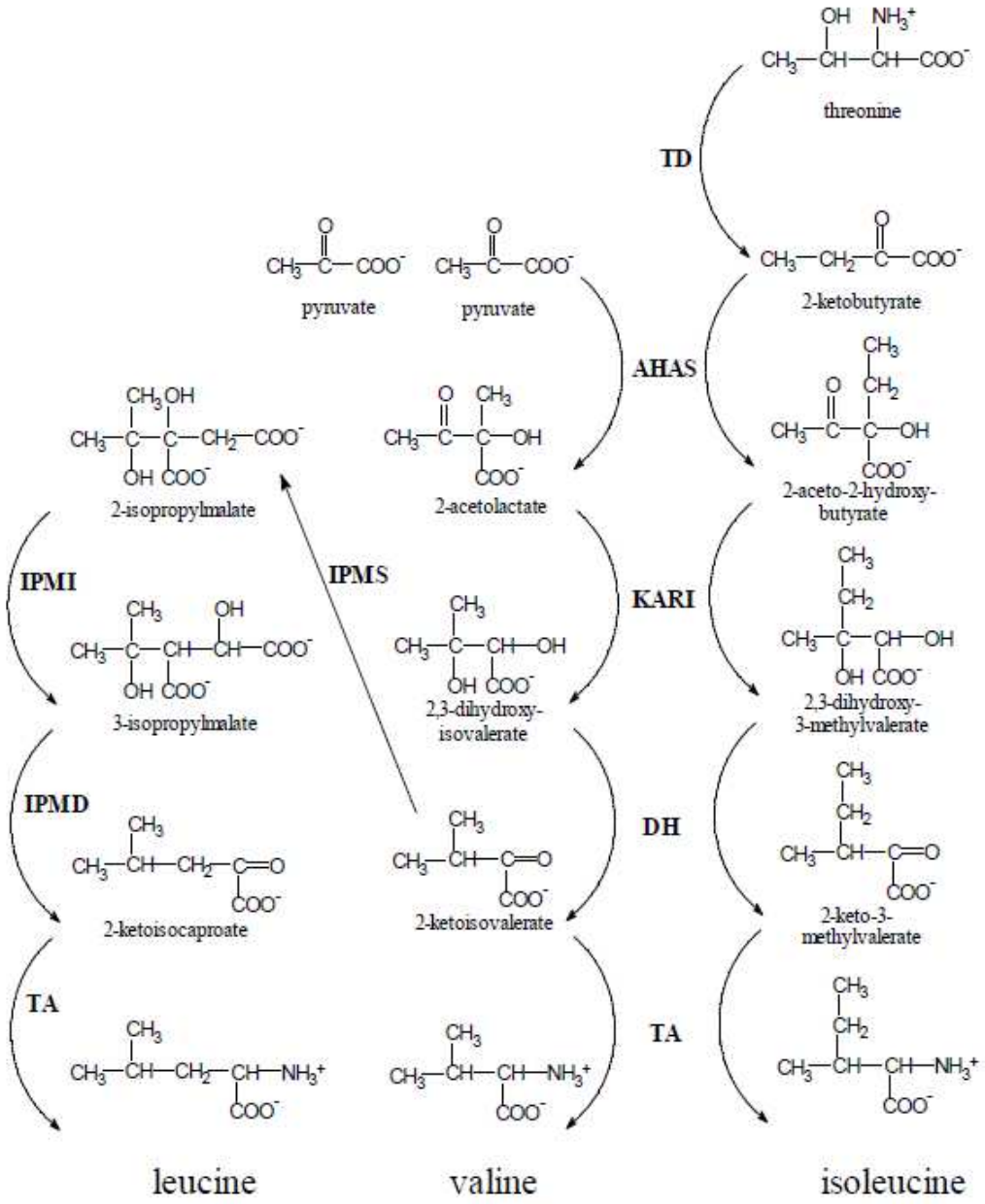


Figure 4. Biosynthetic pathway of branched chain amino acids. Abbreviations used are: TD, threonine deaminase; KARI, ketol-acid reductoisomerase; DH, dihydroxyacid dehydratase; TA, transaminase; IPMS, 2-isopropylmalate synthase; IPMI, isopropylmalate isomerase; IPMD, 3-isopropylmalate dehydrogenase (adopted from Duggleby and Pang, 2000).

Analysis of the crystal structure of yeast AHAS and other studies suggested that AHAS enzyme of all eukaryotes is composed of a large catalytic subunit (Figure 5) and a small regulatory subunit (Duggleby and Pang, 2000; Hershey *et al.*, 1999; Lee and Duggleby, 2002; Pang *et al.*, 2002). The main AHAS enzymatic activity is exerted by the catalytic subunit (Duggleby and Pang, 2000) which forms a homo-dimer, and each catalytic subunit contains three domains, alpha, beta and gamma (Figure 5) (Bekkaoui *et al*., 1993; Duggleby *et al*., 2003; Ott *et al*., 1996; Pang *et al.*, 2002). The AHAS small subunit confers valine sensitivity to the enzyme, therefore it is referred as the regulatory subunit (De Felice *et al.*, 1974; Eoyang and Silverman, 1986; Sella *et al*., 1993; Weinstock *et al.*, 1992; Vyazmensky *et al*., 1996). The regulatory subunit controls AHAS enzyme activity by conferring end-product feedback inhibition, or by increasing specific activity and stability of the catalytic subunit substantially (Duggleby and Pang, 2000).

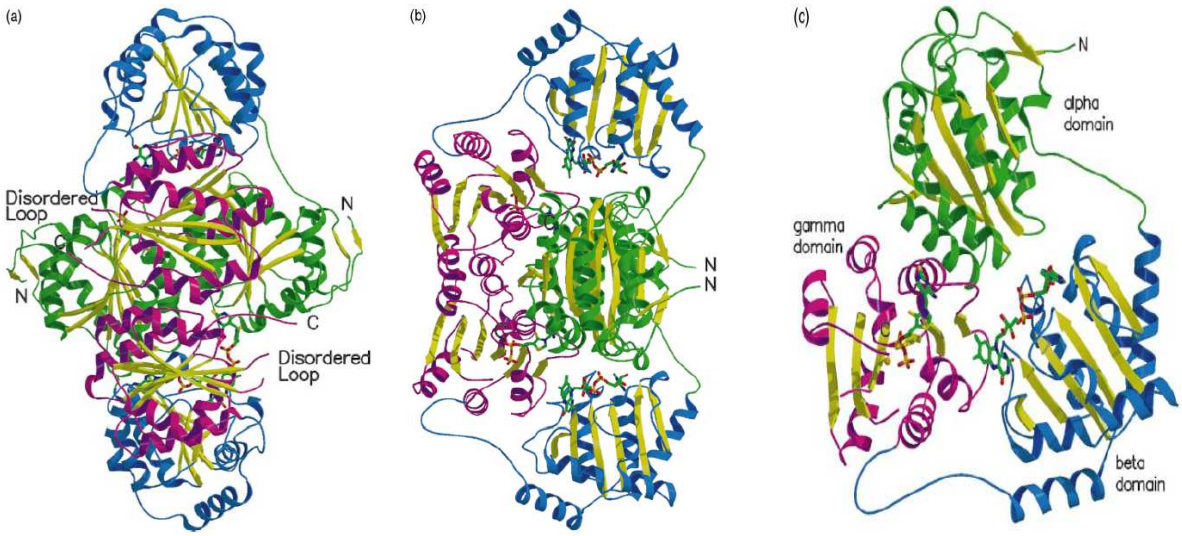


Figure 5. Structure of yeast AHAS. (a, b) are orthogonal views of the catalytic subunit of dimeric yeast AHAS. The alpha-domains are colored green, beta-domains blue and gamma-domains magenta; beta-strands in all domains are colored yellow. FAD (flavin adenine dinucleotide) and

ThDP (thiamin diphosphate) molecules are shown as stick models. (c) is the structure of monomer A of yeast AHAS with alpha, beta and gamma domains (adopted from Pang *et al.*, 2002).

Due to the critical role in ensuring a balanced supply of the amino acids as well as producing intermediates to interact with other cellular metabolic pathways, AHAS enzyme activity is carefully regulated by various mechanisms (Duggleby and Pang, 2000). As mentioned before, one of the mechanisms regulating AHAS activity by end-product feedback inhibition is carried out by the regulatory subunit of AHAS enzyme. Almost all AHAS can be inhibited by at least one of the branched chain amino acids, and valine is clearly the most potent inhibitor in microorganisms and plants. Leucine is an equally good or sometimes better inhibitor than valine (Duggleby and Pang, 2000; Durner and Böger, 1988; Miflin, 1971; Singh *et al*., 1988; Southan and Copeland, 1996). The other mechanism involves the control of the enzyme at the transcriptional level. In plants, at least one AHAS gene is expressed in a constitutive manner but the expression level may vary between tissues and developmental stages. The highest level of AHAS transcription and activity is observed in the metabolically active meristematic tissues (Duggleby and Pang, 2000; Keeler *et al.*, 1993; Ouellet *et al*., 1992; Schmitt and Singh, 1990). Some plants may possess multiple AHAS genes, two of which are housekeeping and other AHAS genes are only expressed in a tissue specific manner (Duggleby and Pang, 2000). AHAS genes have been identified and sequenced in a variety of plants and microorganisms. Duggleby and Pang (2000) have found that a majority of AHAS genes shares up to 73 conserved residues after examining the overall alignment of 24 AHAS sequences from various organisms. However, the function of these residues has never been directly tested or fully understood except being

deduced by analogy with related enzymes. Roles of some important residues of the AHASs from Arabidopsis, yeast and *E.coli* are provided in Table 3.

Table 3. Important residues and their roles in AHASs from Arabidopsis, yeast and *E.coli* (isozyme II) (adopted from Duggleby and Pang, 2000).

Arabidopsis thaliana	***Saccharomyces cerevisiae***	***Escherichia coli* II**	**Role**
A122	A117	A26	Herbicide resistance
E144	E139	E47	Catalysis
P197	P192	S100	Herbicide resistance
A205	A200	A108	Herbicide resistance
G350	G353	G249	FAD binding
W491	W503	W381	FAD binding
M513	M525	M403	ThDP conformation
D538	D550	D428	Mg2+ binding
N565	N557	N455	Mg2+ binding
H567	E579	R457	Mg2+ binding
W574	W586	W464	Herbicide resistance, Substrate specificity
S653	G657	P536	Herbicide resistance

Interestingly enough, although AHAS sequences from different species share a large number of amino acid identities, AHAS sequences from plants and some fungi are observed to be substantially longer than other microorganisms due to an N-terminal extension. AHAS enzyme is normally located in plastids for plants or mitochondria for fungi; that is, it must be transported to these organelles after the enzyme is synthesized. Therefore, N-terminal extension is probably involved in the intracellular trafficking of an AHAS enzyme (Duggleby and Pang,

2000). Also, amino acid composition of the N-terminal extension with a high number of serine residues is a typical feature of chloroplast and mitochondrial transit peptides (von Heinjie *et al.* 1989). The function of the transit peptide is to guide the protein to the target organelle and it is cleaved off during or after translocation. However, the actual cleavage site has not been established for any AHAS protein (Duggleby and Pang, 2000). Many experimental evidences indicate that the N-terminal extension is non-essential for AHAS activity; in fact, removing part or all of the transit peptide sequence is crucial for expression of plant AHAS enzyme in a recombinant system in microorganisms (Chang and Duggleby, 1997; Chang *et al.*, 1997; Duggleby and Pang, 2000; Dumas *et al.*, 1997; Pang and Duggleby, 1999; Wiersma *et al.*, 1990). Also, plant AHAS genes are expected to encode polypeptides with a molecular mass of about 72 kDa, which is roughly 10 kDa larger than a bacterial AHAS catalytic subunit. However, the mature AHAS protein with only 65 kDa mass or less is found in a variety of monocotyledonous and dicotyledonous plant species; meaning the extra 10 kDa is possibly contributed by the N-terminal organelle targeting sequence (Duggleby and Pang, 2000).

An AHAS enzyme requires thiamine diphosphate (ThDP), flavin adenine dinucleotide (FAD) and a divalent metal ion as cofactors to catalyze the initial decarboxylation of pyruvate (Duggleby and Pang, 2000; Halpern and Umbarger, 1959; Störmer and Umbarger, 1964). ThDP is essential for AHAS activity from all species. All ThDP-dependent enzymes contain a conserved 29-32 amino acid motif which begins with the triplet amino acids GDG and ends with NN to interact with ThDP. With no exception, an AHAS also contains exactly the same motif (Candy and Duggleby, 1998; Duggleby and Pang, 2000; Hawkins *et al.*, 1989). The role of ThDP is to break the bond between keto group and carboxyl group carbons of pyruvate to form an intermediate product. The intermediate condenses with the 2-ketoacid substrate to the end

product while ThDP is regenerated (Figure 6) (Duggleby and Pang, 2000). FAD is required for AHAS activity, but its role is not fully understood yet. Two hypotheses of FAD's role have been proposed: the first is that FAD supports the structure of the enzyme in order to maintain the correct geometry for substrate binding and catalytic activity (Duggleby and Pang, 2000). The second hypothesis is that FAD protects α-caranion (Figure 6, V) from protonation during the binding process of 2-ketoacid substrate by allowing the enamine to form a reversible adduct with FAD (Figure 7) (Schloss and Aulabaugh, 1988).

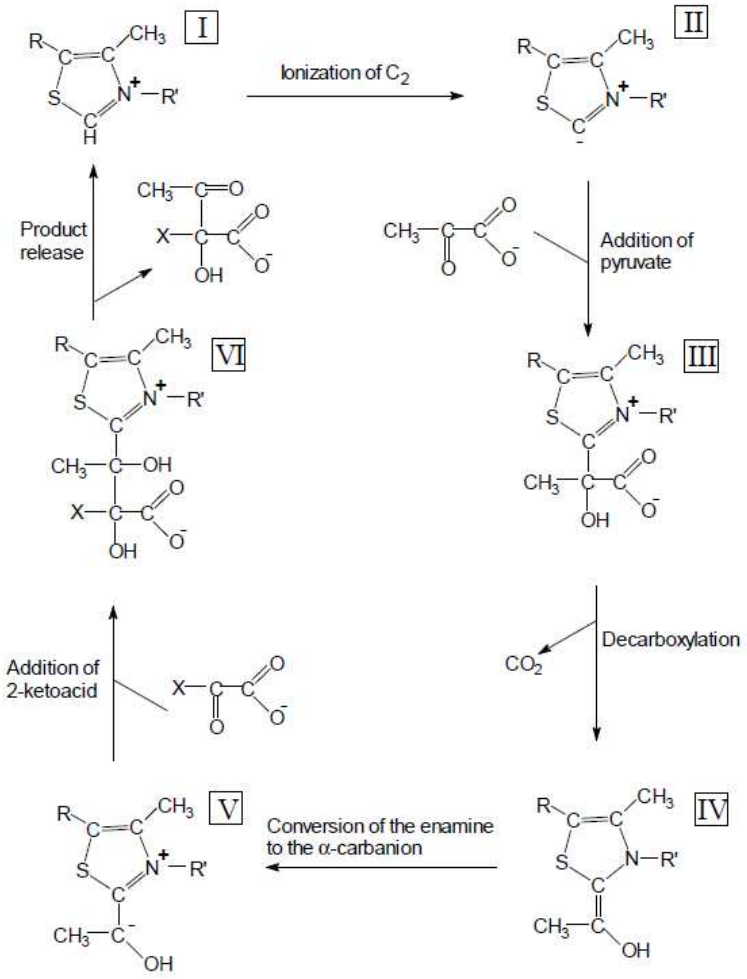


Figure 6. Proposed role of ThDP in catalytic cycle of AHAS. (I) is ThDP in protonated form; (II) is ionized ThDP; (III - VI) are intermediate products (adopted from Duggleby and Pang, 2000).

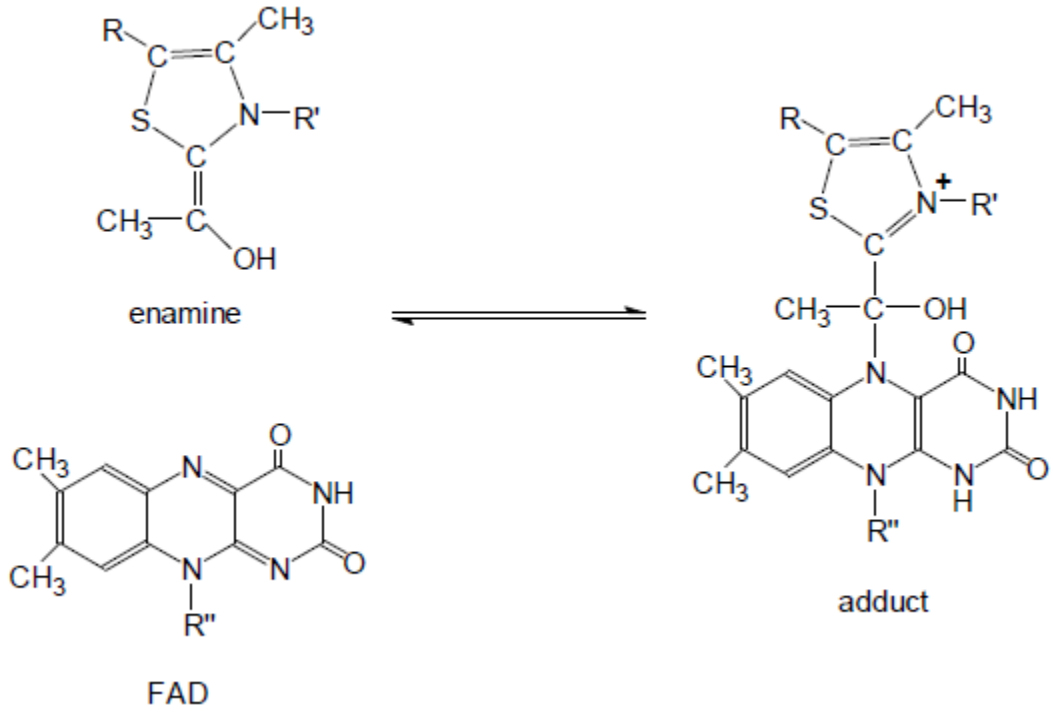


Figure 7. Proposed structure of the enamine-FAD adduct of AHAS by Schloss and Aulabaugh (1988) (adopted from Duggleby and Pang, 2000).

Metal ions are commonly required by all ThDP-dependent enzymes including AHAS for activity, and the requirement is generally satisfied with Mg^{2+}. The role of the metal ion is to act as an anchor to hold the ThDP in place by coordinating it to two of the phosphate oxygen atoms from ThDP and two amino acid side chains from the ThDP-motif of an AHAS (Figure 8) (Duggleby and Pang, 2000; Hawkins *et al.*, 1989). These cofactors are essential for AHAS activity, so they are also required for enzymatic assays of AHAS activity (Singh *et al.*, 1988).

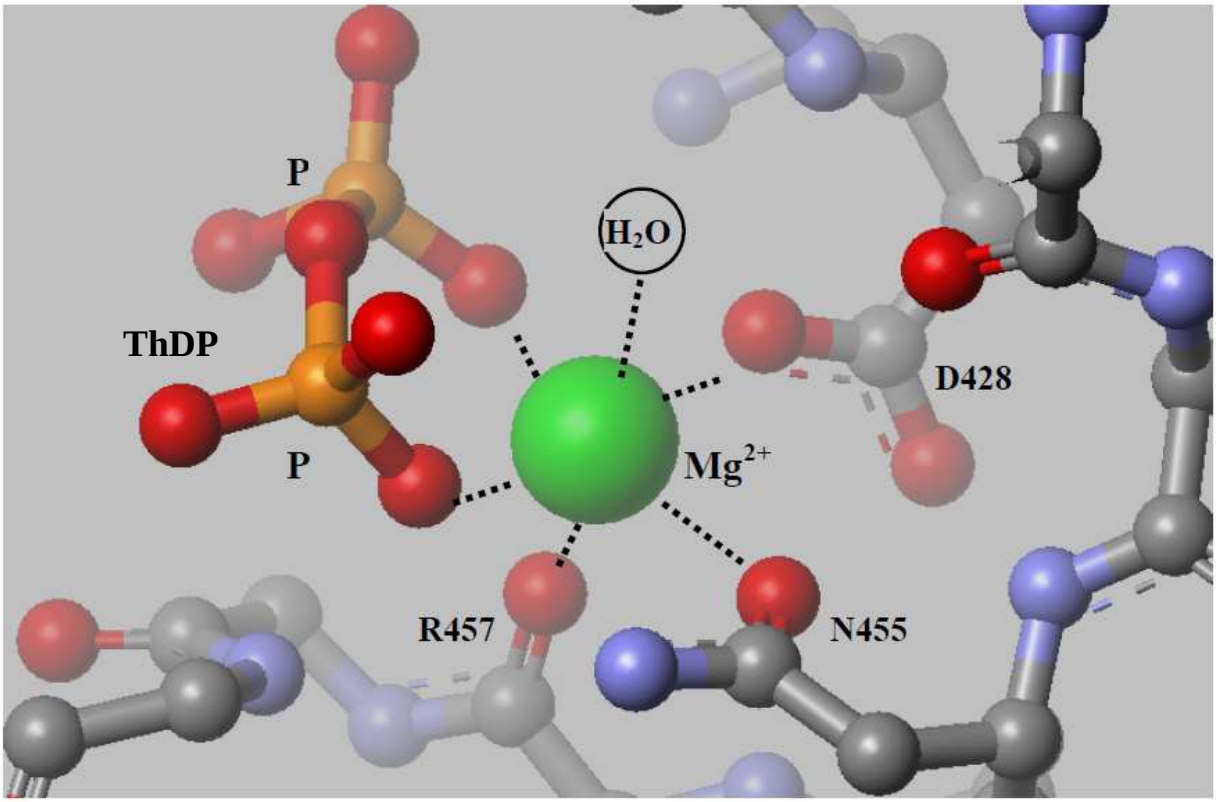


Figure 8. Structural image of a proposed state of a metal ion (Mg^{2+}) anchoring ThDP to ThDP-motif of an AHAS protein (adopted from Duggleby and Pang, 2000).

In most of the studies on AHAS, the enzyme activity is measured using a discontinuous colorimetric assay based on the method developed by Singh *et al.* (1988). In the method, the sample containing AHAS enzyme is incubated for a fixed time between 30 minutes to 2 hours with pyruvate and other additives (including those cofactors). ThDP is included at a concentration of 50 µM at least or more; the metal ion is usually required at a concentration of 0.1 to 10 mM; and FAD is added at a concentration of 2 to 100 µM. The reaction is then terminated by adding sulfuric acid and heated at 60°C for 15 minutes to convert acetolactate to acetoin. By reacting with creatine and α-naphthol, acetoin is converted to a pink-colored complex which can be measured at 520 nm wavelength in a spectrometer. As a result, AHAS

activity can be estimated based on the color intensity (Duggleby and Pang, 2000; Singh *et al*., 1988; Westerfeld, 1945). In order to maintain high activity and stability of the enzyme during series of treatments in an assay, a high concentration of potassium phosphate is recommended at optimal pH 7.0 - 7.5 in the extraction buffer (Duggleby and Pang, 2000; Kuwana and Date, 1975). In addition, high concentrations of glycerol and polyvinylpolypyrolidone (PVPP) have also been reported to help with stabilization of the enzyme in the assay (Durner and Böger, 1988; Muhitch *et al.*, 1987; Relton *et al.*, 1986; Southan and Copeland, 1996). The great advantage of the method is the excellent sensitivity and it can measure as low as 0.0001 units of the enzyme activity routinely.

3.5 Development of imidazolinones resistant crops

Herbicide resistant crops can be developed by traditional breeding and transgenic approach which alters target gene and/or detoxifies a herbicide through metabolism in crops (Duke *et al*., 2002; Kirkwood, 2002; Duke, 2005; Green and Owen, 2011). Alteration of a target gene is to change amino acids of the encoded enzyme resulting in being less sensitive or insensitive to a herbicide, while detoxification of a herbicide is to utilize a specific enzyme to metabolize the chemical before it reaches the target site (Tan *et al*., 2006). By employing these breeding techniques, imidazolinone resistant crops have been developed (Duke, 2005; Tan *et al*., 2006) to benefit weed-management. In general, herbicide resistant crops are free from chemical injury and allow growers to implement flexible and easy management strategy. Growers can select new herbicide options which are environmentally friendly with improved weed control efficacy. Compared to conventional crops, herbicide resistant crops can employ flexible timing of weed control and reduce the cost in the weed management (Madsen and Streibig, 2003; Green and Owen, 2011). In addition to the benefit for farmers, herbicide resistant corps can help to

prevent soil erosion from tillage practice because they need no tillage or less tillage for weed control (Duke, 2001).

Imidazolinone resistant crops have been developed through natural selection of AHAS gene variants or chemical mutagenesis, so the AHAS enzyme becomes less sensitive to imidazolinone herbicides (Newhouse *et al.*, 1991; Shaner *et al*., 1996; Tan *et al*., 2005). In reference to *Arabidopsis thaliana* L., five commonly occurring mutations in the AHAS catalytic subunit at Ala122, Pro197, Ala205, Trp574 and Ser653 contribute to tolerance to AHAS inhibitors (Gressel, 2002; Tranel and Wright, 2002; Christoffers *et al*., 2004; Tan *et al.* 2005). This is because they are located closely within the adjacent area of the protein to form a pocket (Figure 9) where the binding site of AHAS inhibitors is located (Ott *et al.* 1996; Tan *et al.* 2006). Based on molecular modeling of the interaction between AHAS and imidazolinones, the binding pocket (Figure 9) is believed as the entry site of the substrate for an AHAS enzyme (Ott et al., 1996). Thereby, once imidazolinones enter the substrate access channel, they will impede the binding of the substrate to AHAS resulting in loss of activity (Duggleby *et al*., 2003; Pang *et al.*, 2002; Pang *et al*., 2003).

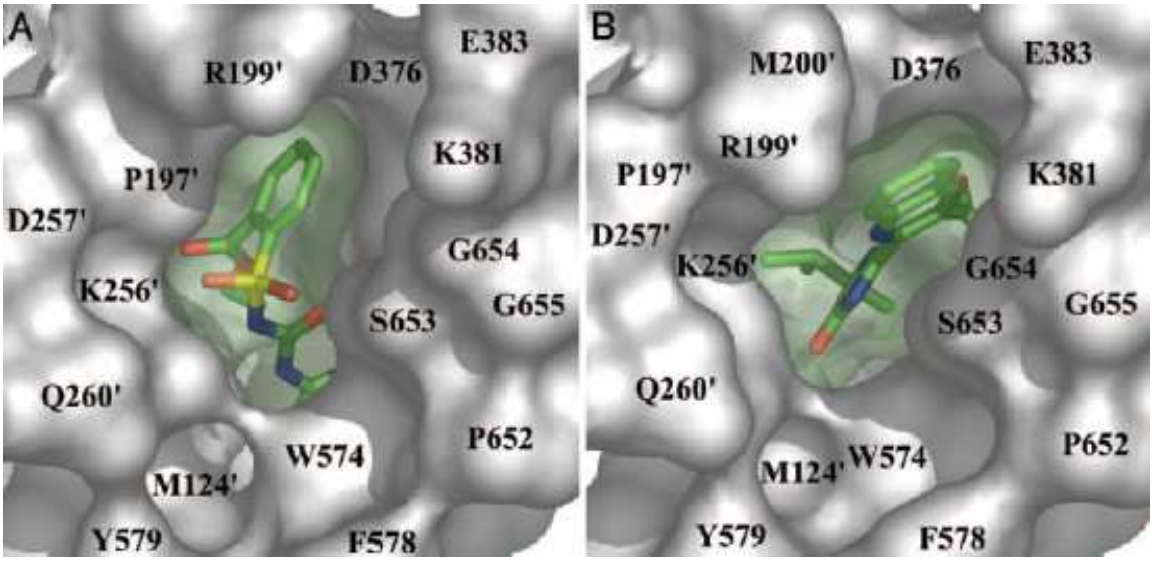


Figure 9. Group 2 herbicides, (A) chlorimuron ethyl and (B) imazaquin, are blocking the binding pocket of the active site channel of *Arabidopsis* AHAS enzyme. The residues that line the binding pocket are depicted as a gray surface. (') indicates residues from the neighboring subunit (adopted from McCourt *et al*., 2006).

Among the five common mutations, Trp574 mutation leads to tolerance to all families of group 2 herbicides (Tan *et al.* 2005); mutation at Pro197 is only tolerant to sulfonylureas and mutations at Ala122, Ala205 and Ser653 are more tolerant to imidazolinones. The Ser653 mutation confers strong tolerance to imidazolinones, but not cross-tolerance to other chemical families in group 2 herbicides, which is preferable for the development of imidazolinone resistant crops (Dietrich, 1998; Lee *et al*., 1999; Sathasivan *et al*., 1991; Tan *et al*. 2006; Tranel and Wright, 2002; Tranel *et al*., 2003). Since 1992, many staple crops, including maize, wheat, rice, canola and sunflower, with imidazolinone-resistance trait, have been developed and commercialized through selection or mutagenesis by utilizing conventional plant breeding techniques.

3.6 Chemical mutagenesis

Ethyl methanesulfonate (EMS) (Figure 10) as alkylating agent is able to induce

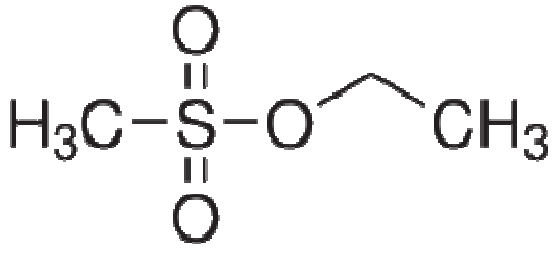


Figure 10. Chemical structure of ethyl methanesulfonate (Sigma-aldrich, 2012)

chemical modification of nucleotides resulting in mismatching and base changes (Kim *et al.* 2006). Under the effect of EMS, guanine (G) is underlying alkylation resulting in forming O^6-ethylguanine, which prefers thymine (T) to cytosine (C) pairing during DNA synthesis; thereby the original G/C pair is replaced by A/T (Greene *et al.* 2003). Consequently, the nucleotide substitution leads to change of the amino acid resulting in insensitivity of the enzyme to the herbicide.

In order to be efficient to produce mutant population, EMS mutagenesis must reach an optimized balance of relatively high mutation rate and minimized sterility in M1 (EMS-treated seeds) and M2 (M1 offspring) generations. However, it is difficult to achieve the balance because of limited information available for EMS concentration and the lethal dose for different plant species (Hohmann *et al.* 2005). Normally high dosage causes a strong mutation rate; but it also increases unwanted mutations on various loci leading to a high rate of sterility or even lethality. To determine the efficacy of chemical mutagenesis, two criteria must be considered: the ratio of sterility, and pigment defects in M1 plants. The sterility of M1 plants is supposed to be significant after an effective treatment, that is, 20 - 50% of M1 plants should have no offspring. The pigment defect ratio should be up to 1% of M1 plants (Koornneef, 2002). In addition to the mutagen dose, the duration of chemical treatment is another factor affecting chemical mutagenesis (Koornneef *et al.* 1982). Although the mutation occurs to M1 plants, the mutant phenotype may not be shown because most mutations are genetically recessive and M1 generation is usually heterozygous for the mutations (Koornneef, 2002). Self-pollination of M1 plants is required to produce M2 seeds (De Haro and Del Rio, 1998), in which heterozygous mutations will segregate resulting in variations in mutant phenotypes (Koornneef, 2002). Therefore, M2 borage plants are used to screen for imidazolinone resistance.

3.7 Kompetitive Allele Specific PCR (KASP) Marker

Single nucleotide polymorphism (SNP) means a single base change in a DNA sequence, with a usual alternative of two possible nucleotides at a given position (Vignal *et al*. 2002). SNPs can occur when a single nucleotide in the genome or other homologous sequences differs between members of a species or between paired chromosomes in an individual (Semagn *et al*. 2014). SNPs have gradually emerged as very powerful and useful tools for various genetic applications, including germplasm characterization, quality control analysis, linkage mapping, allele mining, marker-assisted backcrossing, marker-assistant recurrent selection and genomic selection (Semagn *et al*. 2014). Therefore, high-throughput SNP genotyping technologies and platforms are in demand.

KASP genotyping system (Figure 11) is a fluorescence-based genotyping technology, initially developed by KBioscience for in-house genotyping and eventually evolving into a global benchmark technology (Semagn *et al*. 2014). The technology is based on allele-specific oligo extension and fluorescence resonance energy transfer (FRET) for signal generation (Kumpatla *et al*. 2012).

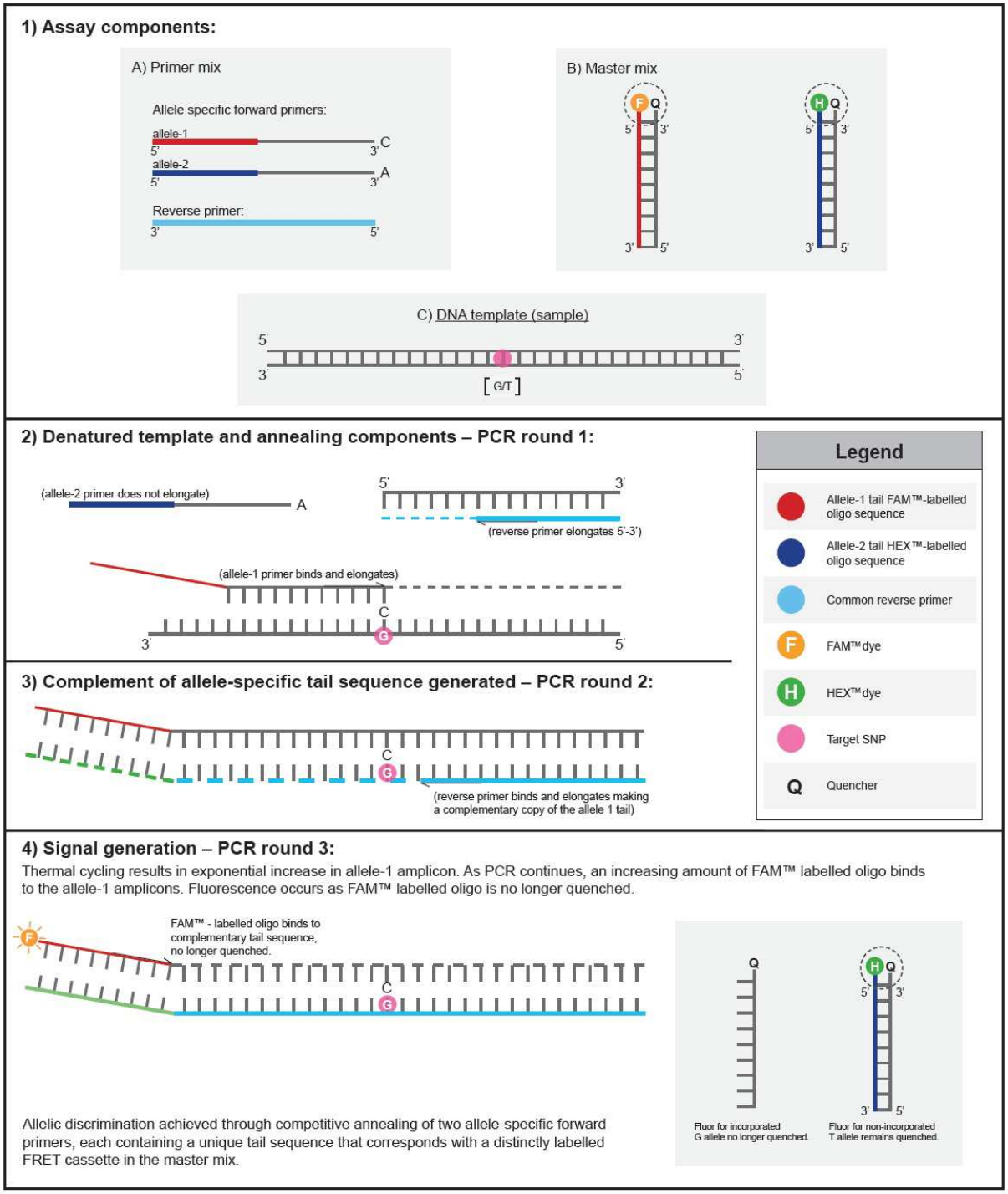


Figure 11. The mechanism of KASP genotyping system (adopted from LGC Genomics, 2014).

KASP genotyping can determine both SNPs and insertion/deletion genotypes by carrying out analysis in 96, 384 and 1536-well plate formats (LGC Genomics, 2014). The assay components

of KASP genotyping contain primer mix, master mix and DNA template (Figure 11-1). Primer mix includes two allele-specific primers and one allele common primer. Each allele-specific primer must be ended with a specific nucleotide of SNP and attached with unique unlabeled tail sequences at the 5' end. The master mix is a mixture of FAM and HEX dye specific FRET cassette, Taq polymerase specially modified for allele-specific PCR and optimized buffer (LGC Genomics, 2014).

When all assay components are combined, the PCR reaction proceeds. The recommended amount of DNA to use per reaction is 5 - 50 ng, and the total reaction volume for the 96-well assay is 10 μL. KASP genotyping should be carried out with at least 22 samples plus two no template controls to ensure there are enough genotypes to show clustering (LGC Genomics, 2014). The mechanism of KASP genotyping involves template denaturation and annealing, formation of the complement of allele-specific tail sequence, and signal generation (Figure 11). In the first PCR cycle, the template denatures resulting in single strand DNA, and then allele-specific primer and common primer bind to the single stand DNA to amplify new double-strand DNA products (Figure 11-2). In the following cycle, after DNA products denature, a common primer will bind to a single strand DNA to synthesize the complementary strand DNA including the complement of allele-specific tail sequence (Figure 11-3). In the next cycle, dye-labeled oligo sequence from the master mix will only anneal with those single strand DNA containing the complement of allele-specific tail sequence resulting in fluorescent signal (Figure 11-4). According to the signal generated (either one type or a mixture of two types), genetic materials can be assigned to homozygous and heterozygous genotypes.

KASP can be used for genotyping a wide range of germplasm for various purposes including quality control analysis, linkage mapping, marker-assistant recurrent selection and

allele mining applications that require SNP data ranging from a few to several hundreds of data points per sample (Semagn *et al*., 2014). Compared with other genotyping platforms, the KASP genotyping has lower occurrence of false signals, lower cost in identifying differences in the genotypes and offers scalable flexibility in applications that require small to moderate numbers of markers (Semagn *et al*., 2014).

4.0 RESEARCH STUDIES

4.1 Study 1: EMS mutagenesis and screening of the mutant population for herbicide-resistance

4.1.1 Abstract

Different concentrations of EMS were used to treat M1 borage seeds and the mutagenized seeds were then grown to maturity in the field to obtain M2 seeds. M2 plants were screened for imidazolinone tolerance and survived individuals were selected and self-pollinated manually to produce M3 seeds. The M3 plants were subjected to herbicide screening again to confirm the phenotype. This process resulted in identification of two stable imidazolinone resistant lines at different phases of the project.

4.1.2 Hypothesis

EMS induces a point mutation of genomic DNA through single nucleotide substitution from G/C to A/T. EMS mutagenesis has been used to produce imidazolinone resistant Arabidopsis and other plant species by inducing mutation in AHAS genes. Therefore, this approach could be also used to generate borage mutants that are resistant to imidazolinones.

4.1.3 Introduction

Ethyl methanesulfonate (EMS), a chemical mutagen, can induce nucleotide mismatching and base changes in a genome resulting in genetic mutations (Kim *et al.* 2006). Under the effect of EMS, guanine (G) is undergoing alkylation to form O^6-ethylguanine, which prefers thymine (T) to cytosine (C) pairing during DNA synthesis; thereby the original G/C pair is replaced by A (adenine)/T pair (Greene *et al.* 2003). The nucleotide substitution could lead to changes of amino acids at critical positions resulting in sensitivity variations to herbicides. Although the mutation occurs to M1 plants, mutant phenotypes may or may not be shown in the M1 generation because most mutations are genetically recessive (Koornneef, 2002). Self-pollination of M1 to produce M2 is necessary to allow heterozygous mutants to segregate resulting in variations in mutant phenotypes (De Haro and Del Rio, 1998; Koornneef, 2002), thus M2 plants are screened for herbicide resistance. Many herbicide-resistant crops have been developed using this approach. This study aims to develop imidazolinone herbicide resistant borage through EMS mutagenesis and herbicide screening.

4.1.4 Experimental approach

4.1.4.1: Generation of an EMS mutagenized borage population

Approximately 164000 borage seeds were divided into 6 groups for mutagenesis. The seeds (M1) were soaked in 0.5%, 1.0% and 1.5% (v/v) of EMS solutions for 8 hours and 16 hours respectively, and then rinsed with tap water for 4 hours. After washing, the seeds are dried with paper towel. The mutagenized seeds were sowed in 48 plots in AgQuest research farm, Saskatoon, SK, in June, 2012. Each plot was 7.5 x 1.5 m in size and sowed 86 g of mutagenized seeds. M1 borage plants were grown to maturity and M2 seeds were harvested by groups according to the EMS treatments.

4.1.4.2: Herbicide tolerance screening of mutagenized M2 population and wild-type population

Herbicide tolerance screening was carried out in a growth chamber in the Innovation Place (Saskatoon). M2 seeds were planted at 1 - 2 cm in 25 x 50 cm flats containing commercial potting mix (Sunshine Mix 3; Sun Gro.) in the growth chamber under a 16 hour light (22°C) and 8 hour dark (16°C) cycle. Each flat contained 72 seeds. Group 2 herbicide, "Solo" (BASF Corp.), was applied over foliage when most plants were at the two-leaf stage in a specialized herbicide treatment chamber. The spray solution included 84 g ai/ha (active ingredient per hectare) imazamox with adjuvant Merge (BASF Corp.) at 0.5% (v/v). A moving nozzle cabinet sprayer with a flat-fan nozzle tip was calibrated to deliver 102 L/ha spray solution in a single pass. M2 plants were visually evaluated 21 days after herbicide spray by comparing herbicide treated and untreated wild-type borage controls. Putative tolerant M2 plants were transplanted, self-pollinated and grown to maturity. Their offspring, M3, underwent the same screening process to confirm imidazolinone-resistant phenotype. False positive materials were discarded, and truly tolerant materials were archived. The screening process was carried out continuously until homozygous resistant plant was identified.

Wild-type borage was also screened for imidazolinone resistance. Approximately 14 kg wild-type seeds were sowed in the field in AgQuest research farm, Saskatoon, SK, in June, 2013. "Solo" herbicide containing 84g ai/ha imazamox with adjuvant Merge at 0.5% (v/v) was applied over foliage by a tractor sprayer at a spray rate of 100 L/ha, when most plants were at the 2 - 4 leaf stage. Visual evaluation was initiated after 3 weeks of herbicide application by comparing to non-sprayed wild-type borage. Putative tolerant plants were marked and grown to maturity in the

field. The seeds collected from those putative tolerant plants were subjected to herbicide screening again in the growth chamber.

4.1.5 Results

Generation of a borage mutant population by EMS-induced mutagenesis

Approximately 164000 EMS-treated M1 seeds were sown in the field, of which approximately 20,000 germinated, accounting for about 12% of the germination rate (Table 4). As shown in the table, lower concentration of EMS and shorter period of treatment led to higher rate of germination. From M1 borage, a total of 3.5 kg of M2 seeds were harvested, which constituted a mutant population for imidazolinone resistance screening.

Table 4. The Germination rate of M1 seeds from EMS-induced mutagenesis

Treatment	Germinated M1 seeds	Sown seeds	Germination Rate (%)
0.5% EMS 8hrs	3870	20718	19%
0.5% EMS 16hrs	1728	20718	8%
1.0% EMS 8hrs	6104	41436	15%
1.0% EMS 16hrs	3586	41436	9%
1.5% EMS 8hrs	2273	20718	11%
1.5% EMS 16hrs	1602	20718	8%

Phenotypic observation of mutant plants

Phenotypic survey of M1 plants in the field observed many unusual morphological changes. For instance, a normal borage flower has 5 petals, while the abnormal number of petals

such as 4 or 6 in flowers was seen in the mutant plants (Figure 12). In addition, dwarf and delayed growth and development plants were frequently observed (Figure 12).

Figure 12. Mutant phenotypes of the M1 borage population. Top row: flowers with abnormal 4 and 6 petals; bottom row: plants with dwarf, delayed growth phenotypes.

Screening of the mutant population for imidazolinone resistance

Imidazolinone resistance screening was carried out in a specialized spraying chamber equipped with a moving nozzle herbicide sprayer. About 2X of agronomically recommended dosage of “Solo” herbicide was applied to M2 borage plants. The screening resulted in identification of the first tolerant plants (Figure 13, left) from offspring of the M1 seeds that were treated with 1.5% EMS for 16 hours. This plant was then selfed and kept growing to maturity. A total of 271 M3 seeds was harvested from the tolerant plant, and subjected to herbicide screening

again. Altogether 225 of 271 M3 seeds were germinated. After herbicide screening by 2X of "Solo" herbicide, 169 plants survived and 56 were killed by the herbicide. The ratio of the imidazolinone-tolerant and imidazolinone-susceptible of the M3 borage plants was 3:1 [X^2 (1, N = 225) = 0.001, p < 0.01]. From those survived plants, 9 of them were transplanted and pollinated by hand to produce M4 seeds. Using the similar screening procedure, the second imidazolinone tolerant borage plant (Figure 13, right) was identified in the field.

Figure 13. Screening for imidazolinone resistant plants. Left picture: the first tolerant borage plant (M2) (an *AHAS1* mutant); right picture: the second imidazolinone tolerant borage plant (an *AHAS2* mutant).

4.1.6 Discussion

EMS has been widely used for generating genetic mutants in various crop species. Efficacy of EMS mutagenesis is dependent on concentration and duration of treatment. Due to the large seed size and thick seed coat, borage seeds require a higher concentration of EMS than those crops with small seeds. De Haro-Bailóna and Del Riob (1998) used 1.0% (v/v) of EMS to soak borage seeds for 16 hours for the mutagenesis. In this study, three different EMS

concentrations from 0.5% to 1.5% and two different time lengths were used for mutagenesis. Among 6 groups of treated M1 seeds, the germination rate ranged from 8% to 19%, which was significantly lower than normal germination rate (approximately 40 - 60%) (Wu, 2012). For 8 hour treatments, seeds exposed to higher concentration of EMS had lower germination rate; while for 16 hour treatments, germination rates were similar at 8% to 9%, despite of different EMS treatment concentrations. Lower rate of germination was observed to associate with longer time of EMS exposure in the study. In addition, pronounced sterility was observed in the group of M1 seeds soaked in 1.5% EMS for 16 hours. The amount of M2 seeds harvested from the group was extremely small, only 65 g in 3.5 Kg of M2 seeds sown. However, it is noted that the first imidazolinone tolerant borage plant was identified from this group of mutant seeds.

All M1 and selected M2 tolerant plants did not show any change of flowers color; however in selected M3 plants, 5 out of 9 tolerant plants produced white flowers instead of blue ones. White flowers are occasionally observed from natural mutation in the field; however, blue flower is genetically dominant over white flower (Wu, 2012). EMS-induced mutations could occur randomly at various locations throughout the genome (Greene *et al*., 2003). Thus, mutations can be found not only in the AHAS genes responsible for imidazolinone tolerance, but also on the locus involved in the biosynthesis of flower pigments. As a result, EMS mutagenesis can generate loss-of and gain-of-function mutants at the same time in a single plant (Kim *et al.* 2006). Identified imidazolinone tolerant borage, thereby, may lose certain good traits after mutagenesis. Further breeding is thus required and highly necessary to integrate imidazolinone-resistance trait into a commercial borage line.

EMS mutagenesis can introduce a single nucleotide substitution of one AHAS allele in M1 plants. This means that M2 would be a segregation population on the gene. As AHAS

mutation is commonly dominant or semi-dominant, AHAS heterozygous mutant in the population can show the tolerant phenotype to imidazolinones (Duggleby and Pang, 2000; Tar'an, personal communication). Homozygous imidazolinone-resistant plants may appear more tolerant than heterozygous individuals, but it is still difficult to distinguish them by visual screening inspection. Therefore, a genotyping marker is needed to identify homogeneity of the mutant allele.

4.2 Study 2: Cloning of borage AHAS genes and identification of point mutation responsible for imidazolinone resistance

4.2.1 Abstract

AHAS protein sequences of Arabidopsis and sunflower were used as queries to BLAST search a partial genome database of borage resulting in identification of many short fragments of DNA sequences that were homologous to the AHAS sequences. Based on the fragment sequences, several sets of primers were designed to retrieve missing ends of borage AHAS genes by RACE-PCR. After retrieving the missing ends, specific 5' and 3' end primers were designed to obtain full length AHAS genes. By this way, two homologous AHAS genes, *AHAS1* and *AHAS2*, were cloned from wild-type and imidazolinone-resistant borage plants, respectively. Comparison of these sequences revealed point mutations in two AHAS genes responsible for imidazolinone resistance.

4.2.2 Hypothesis

AHAS is a house-keeping gene in living organisms essential for the biosynthesis of branched amino acids. Plant species share high homology of AHAS protein sequences. Using previously identified AHAS protein sequences from the other species to search a borage partial

genome sequence database would enable identification of partial sequences of borage AHAS genes. RACE-PCR can then be used to obtain the the full length of borage AHAS genes.

4.2.3 Introduction

Acetohydroxyacid synthase (AHAS) (EC 4.1.3.18), as known as acetolactate synthase (ALS), catalyzes the first reaction in the pathway for synthesis of branched chain amino acids leucine, isoleucine and valine in plants and microorganisms (Duggleby and Pang, 2000). AHAS plays a critical role to ensure a balance supply of the amino acids as well as producing intermediates to interact with other cellular metabolic pathways (Duggleby and Pang, 2000). In plants, the gene encoding AHAS enzyme, *AHAS*, is generally expressed in a constitutive manner, but expression level may vary between tissues and developmental stages (Duggleby and Pang, 2000; Keeler *et al.*, 1993; Ouellet *et al*., 1992; Schmitt and Singh, 1990). The identity of AHAS protein sequences among different species ranges from 17% to 90%, and many key residues of AHAS enzymes are absolutely conserved across species (Duggleby and Pang, 2000). Imidazolinone herbicides control weeds by inhibiting activity of native AHAS enzyme (Tan *et al*., 2005), thus natural mutation or chemical induced mutations in AHAS gene could result in the enzyme with less or no sensitivity to imidazolinone herbicides (Newhouse *et al.*, 1991; Shaner *et al*., 1996; Tan *et al*., 2005). In reference to *Arabidopsis thaliana*, five commonly occurring mutations in a AHAS gene for the catalytic subunit at codon locations of Ala122, Pro197, Ala205, Trp574 and Ser653 contribute to tolerance to AHAS inhibitors (Gressel, 2002; Tranel and Wright, 2002; Christoffers *et al*., 2004; Tan *et al.* 2005). This study aims to clone AHAS genes from the wild-type and the mutant borage plants, and to identify the mutation responsible for the herbicide resistant phenotype.

4.2.4 Experimental approach

4.2.4.1: RNA isolation, RACE-Ready cDNA Synthesis

Total RNA was extracted from borage leaves. About 0.5 - 1.0 g leaf tissue was pulverized in liquid nitrogen to fine powder using pestle and mortar. Total RNA was isolated using 1 mL Trizol reagent (Invitrogen Corp) per 50-100mg of tissue sample according to the manufacturer`s recommendation. RNA was quantified by absorbance at 260nm and 280nm using NanoDrop spectrophotometer (NanoDrop Technologies).

RACE-Ready cDNAs including both 5`-RACE-Ready cDNA and 3`-RACE-Ready cDNA were synthesized according to SMARTer RACE cDNA amplification manual (Clontech Laboratories). 3.75 µL mixture of the RNA and 5'-cDNA synthesis primer A were incubated at 72°C for 3 minutes, then cooled to 42°C for 2 min. After cooling, the mixture was briefly centrifuged for 10 seconds at 14,000 g, and then 1.0 µL of the SMARTer IIA oligo were added to 5`-RACE-Ready cDNA synthesis reaction. 4.0 µL buffer mix including 2.0 µL of 5X first strand buffer, 1.0 µL of 20 mM DTT and 1.0 µL of 10 mM dNTPs was combined with 0.25 µL of 40 U/µL RNase inhibitor and 1.0 µL of 100U SMARTScribe reverse transcriptase to form the master mix. The mixtures were incubated in a hot-lid thermal cycler at 42°C for 90 min, and then heat at 70°C for 10 minutes. The RACE-Ready cDNA products were diluted with Tris-EDTA buffer and stored at -20°C. Similarly, 3'-RACE-Ready cDNA was synthesized by the same procedure.

4.2.4.2: Cloning of borage AHAS1 gene

Sunflower AHAS gene was used as a query to blast search against the database of borage partial genomic sequences by CLC workbench software (CLC Bio). Primers were designed upon

the borage DNA fragments that have highest homology with the query sequence. Three reverse primers (AHAS1-5R-R1, AHAS1-5R-R2 and AHAS1-FLR, Table 5) and one forward primer (AHAS1-3RF, Table 5) were designed. 5' prime end RACE-PCR reaction was carried out in 25 µL reaction mixture, containing 3.35 µL molecular biology grade water, 12.5 µL 2X buffer, 2.5 µL of 2 mM dNTPs (Novagen, EMD Chemicals), 2.5 µL Universal Primer A Mix (UPM, ClonTech Laboratories), 1.25 µL primer AHAS1-5R-R1 or AHAS1-5R-R2 respectively, 2.5 µL 5' end cDNA and 0.4 µL KOD Xtreme Hot Start DNA polymerase (Novagen, EMD Chemicals). The PCR profile was as follows: initial denaturation, 94°C for 3 minutes; 3 cycles X (94°C for 30s, 72°C for 80s); 5 cycles X (94°C for 30s, 68°C for 30s, 72°C for 80s); 25 cycles X (94°C for 30s, 63.5°C for 30s, 72°C for 80s); and final extension at 72°C for 10 minutes.

Table 5. Primers for retrieving borage AHAS1 & AHAS2 genes

Primer	Sequence
AHAS1 5RR1	CCAATCATCCTACGAGGTACTTGTCCAG
AHAS1 5RR2	GCAAAAACTCCTCCCTGTTCATGCCTAG
AHAS1 3RF	ACGTGCTTCCTAGGCATGAACAGGGA
AHAS1 FLR	ACACGGTGAACTCGTCTAACCTTGAGGA
AHAS1 FLF	GAAGCCATGGGGATCTCCTCACATTTCACAACC
AHAS2 5RR1	TTGTCCAACACCGGTACTTATGATTGCAT
AHAS2 5RR2	TAGCATCTCCAAACGTTTTAAATGTCAACG
AHAS2 3RF1	TCCTCGTAGATGATTGGTACTGATGCG
AHAS2 3RF2	GCCTGGCCCGGTTTTGATTGACGT
AHAS2 FLR	TGAAATACAACGCAAGTCAAACTCTAC
AHAS2 FLF	TCTCCACCACTCTCTTCACCGTC

The amplification products were resolved on 1% agarose gel. A 1 kb plus DNA ladder was used as a size marker (New England BioLabs). Bands of expected sizes were excised from the gel, and DNA was eluted from the bands using the EZ-10 spin column gel extraction kit following the manufacturer's protocol (Bio Basic Inc.). The eluted DNA was verified by nested RACE-PCR using another reverse primer AHAS1-5R-R2. The nested RACE-PCR reaction contained 2.5 µL Nested Universal Primer A (NUP, ClonTech Laboratories) and 1.25 µL primer AHAS1-5R-R2. After amplification, the products were separated by agarose electrophoresis and one band with correct size was excised from the gel. The DNA was eluted from the band.

To clone the fragment, the 5'-RACE DNA fragment was first extended with poly-A's using Taq polymerase and then 3 µL PCR product was mixed with 5 µL 2X rapid ligation buffer, 1 µL *pGEM-T* vector and 1 µL T4 DNA ligase (Promega Corp). The mixture was incubated at 4°C overnight for ligation. 2 µL of the ligation was transformed into 35 µL of *E. coli* Top Ten cells by electrophoresis. After incubation at 37°C for 1 hour, competent cells were spread onto prepared selecting plates. After incubation at 37°C for 16 to 24 hours, plates were examined for white colonies which are indicative of transformants. White colonies were picked and incubated individually at 37°C for 16 to 24 hours. Concurrently, colony PCR, containing 18.3 µL molecular biology grade water, 2.5 µL 10X buffer, 2.5 µL $MgSO_4$, 0.5 µL of 10mM dNTPs, 0.5 µL NUP, 0.5 µL primer AHAS1-5R-R2 respectively, 0.2 µL Taq polymerase and a dip of colonies as template, were performed to verify the transformants. PCR profile was as follows: initial denaturation, 95°C for 2 minutes; 30 cycles X (95°C for 30s, 62°C for 30s, 72°C for 2 min); and final extension at 72°C for 10 minutes. Plasmid DNAs in positive transformants was isolated and purified using the EZ-10 spin column plasmid DNA kit following the

manufacturer's protocol (Bio Basic Inc.). The DNA was quantified by absorbance at 260nm and 280nm using NanoDrop spectrophotometer before sequencing.

PCR amplification of 3' ends was less complicated because 3' end could be obtained from the borage genomic database. Simple PCR was carried out in 50 µL reaction mixture, containing 33.5 µL of molecular biology grade water, 5 µL 10X Pfu buffer (Bio Basic Inc.), 1 µL of 10 mM dNTPs, 2.5 µL AHAS1-3RF, 2.5 µL AHAS1-FLR, 5 µL cNDA and 0.5 µL Pfu DNA polymerase (Bio Basic Inc.). PCR profile was as follows: initial denaturation, 98°C for 2 minutes; 35 cycles X (98°C for 30s, 68°C for 30s, 72°C for 2 minutes); final extension at 72°C for 10 minutes.

The 5' and 3' ends of AHAS1 gene were assembled using Vector NTI software (Invitrogen). According to the putative full length of *AHAS1*, a forward primer (AHAS1-FLF) from 5' end was designed. Full-length gene was amplified by Pfu PCR reaction in 50 µL reaction mixture, containing 33.5 µL molecular biology grade water, 5 µL 10X phusion buffer, 1 µL of 10 mM dNTPs, 2.5 µL AHAS1-FLF, 2.5 µL AHAS1-FLR, 5 µL cNDA and 0.5 µL Pfu DNA polymerase. PCR profile was as follows: initial denaturation at 98°C for 2 minutes; 35 cycles X (98°C for 30s, 65°C for 30s, 72°C for 2.5 minutes); final extension at 72°C for 10 minutes. Full-length AHAS1 gene was obtained following the same procedure described above including gel DNA extraction, poly-A's overhang, ligation to *pGEM-T* vector, transformation of the vector to *E. coli*, colony selection and plasmid DNA extraction and sequencing. Due to the length of AHAS1 gene, extra sequencing primer, AHAS1 3RF, was used to obtain the middle part of the sequence.

4.2.4.3: Cloning of borage AHAS2 gene

Borage AHAS1 gene was used as a query to BLAST search against the database of borage genomic sequences by CLC workbench software (CLC Bio). Homologous partial sequences were assembled and compared with AHAS1 gene sequence, which indicated the presence of a second AHAS gene in borage. Two forward primers (AHAS2-3RF1 & AHAS2-3RF2, Table 5) and two reverse primers (AHAS2-5RR1 & AHAS2-5RR2, Table 5) were designed for retrieving the AHAS2 gene. Five prime end and 3' end RACE-PCR reactions were carried out in 25 µL reaction mixture, containing 3.35 µL molecular biology grade water, 12.5 µL 2X buffer (Novagen, EMD Chemicals), 2.5 µL of 2 mM dNTPs, 2.5 µL Universal Primer A Mix (UPM, ClonTech Laboratories), 1.25 µL primer AHAS2-5RR1 and AHAS2-5RR2 each (AHAS2-3RF1 and AHAS2-3RF2 for 3'end RACE-PCR), 2.5 µL 5' end cNDA (3' end cNDA for 3'end RACE-PCR) and 0.4 µL KOD Xtreme Hot Start DNA Polymerase (Novagen, EMD Chemicals). PCR profile was as follows: initial denaturation: 94°C for 3 minutes; 3 cycles X (94°C for 30s, 72°C for 80s); 5 cycles X (94°C for 30s, 68°C for 30s, 72°C for 80s); 35 cycles X (94°C for 30s, 63°C for 30s, 72°C for 80s); the final extension at 72°C for 10 minutes. The rest of assembly, amplification and cloning of AHAS2 genes followed the same procedure as described in the section of cloning AHAS1 gene.

4.2.4.4: Identification of point mutations in AHAS genes responsible for imidazolinone resistance

Using the same primer sets, *AHAS1* and *AHAS2* from imidazolinone resistant borage were amplified, cloned and sequenced following the exactly same procedure as described above. By comparing AHAS genes between wild-type and resistant borage plants using Vector NTI

software (Invitrogen), point mutations responsible for imidazolinone resistance were finally identified for both imidazolinone resistant lines.

4.2.5 Results

Using Arabidopsis and sunflower AHAS sequences as queries to search a partial genomic sequence database, two homologous AHAS genes were identified in borage. However, the two borage sequences were not in full-length. To obtain the missing 5' and 3' ends of the AHAS genes, RACE-PCR approach was used to retrieve the sequence information of the missing ends. Analysis of the assembled full-length AHAS1 and AHAS2 genes (Appendix 1) indicated that the open reading frame (ORF) of *AHAS1* was 2007 bp in length encoding a protein of 669 amino acids, and the ORF of *AHAS2* was 1995 bp long encoding a polypeptide of 665 amino acids. Protein sequence comparison of borage and other plants AHASs showed that borage *AHAS1* and *AHAS2* were 95% identical at the amino acid level and both shared approximately 75% of amino acid identity with Arabidopsis AHAS protein and 80% with sunflower AHAS1 protein sequence (Figure 14). The comparison of AHAS genes isolated from the wild-type and two imidazolinone resistant lines revealed that one single nucleotide substitution (from G to A) occurred in AHAS1 gene at 1953bp (Figure 15) in the first resistant line (Appendix 2), which resulted in an amino acid change at position 651 from serine (S) in the wild type to asparagine (N) in the mutant (Figure 16). Interestingly enough, the second resistant line (Appendix 2) has the same single nucleotide substitution in *AHAS2*, but not *AHAS1*, at 1941bp (Figure 15) resulting in the same amino acid change at position 647 (Figure 16).

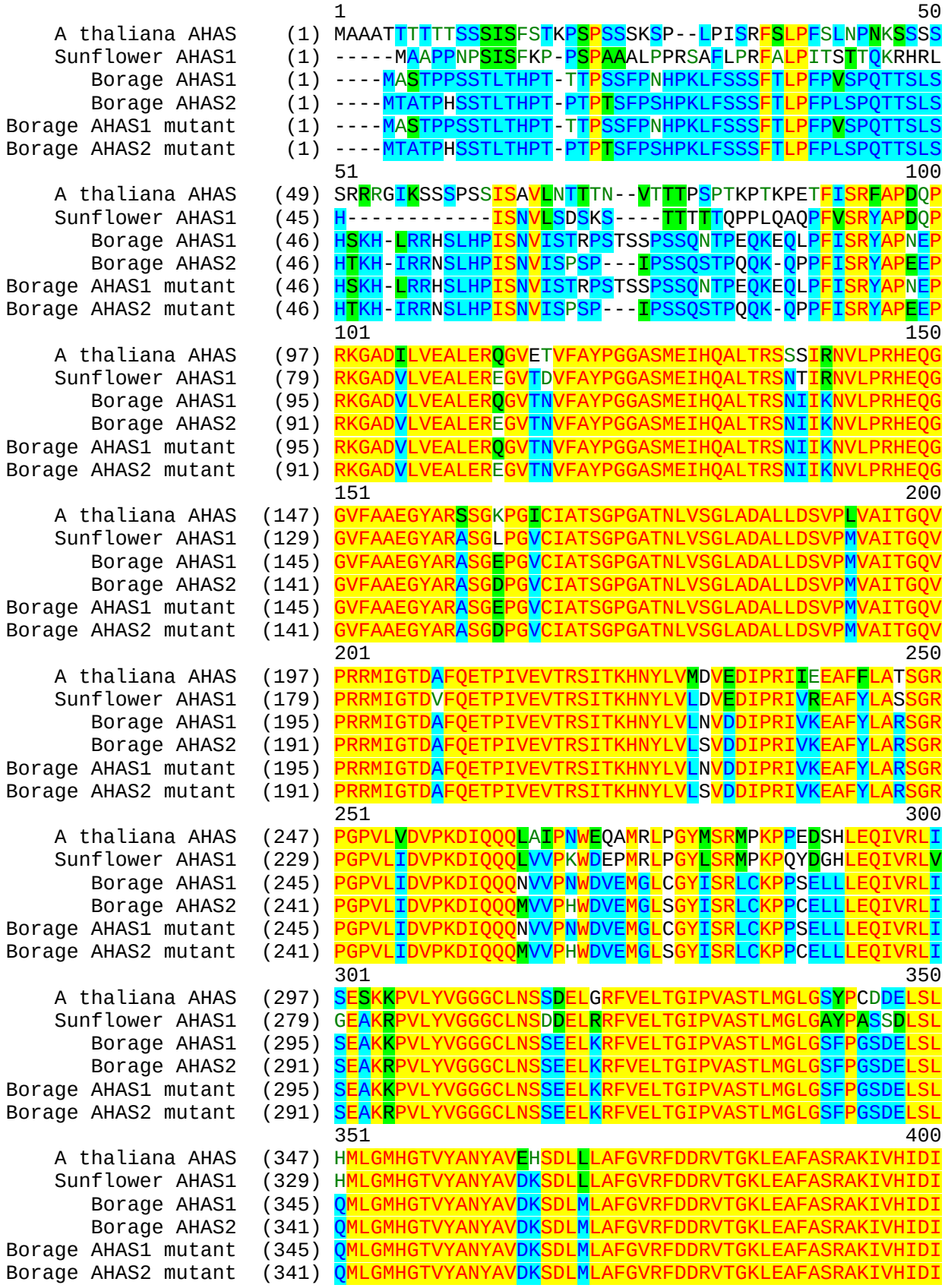
Percent Identity

	1	2	3	4	5	
1		77.7	75.5	75.7	75.4	**1**
2	26.5		80.2	80.7	80.2	**2**
3	29.7	23.0		94.9	99.9	**3**
4	29.4	22.4	5.3		94.7	**4**
5	29.9	23.0	0.1	5.5		**5**
	1	**2**	**3**	**4**	**5**	

1 Arabidopsis AHAS
2 Sunflower AHAS1
3 Borage AHAS1
4 Borage AHAS2
5 Borage AHAS1 mutant

Figure 14. Amino acid identities of AHAS proteins from *Arabidopsis thaliana*, sunflower (*Helianthus annuus*) and borage. The percentage of identities and differences of AHAS proteins are symmetrically displayed across the table.

Figure 15. Alignment of partial sequence of *AHAS1* and *AHAS2* from imidazolinone susceptible and resistant borage. Top two sequences are AHAS genes of susceptible borage. Bottom two sequences are mutant *AHAS1* and *AHAS2* from two resistant borage lines. Single nucleotide substitutions from G to A in mutant *AHAS1* at 1953bp and mutant *AHAS2* at 1941bp were highlighted in the black dashed line box.


1 50
A thaliana AHAS (1) MAAATTTTTTSSSISFSTKPSPSSSKSP--LPISRFSLPFSLNPNKSSSS
Sunflower AHAS1 (1) -----MAAPPNPSISFKP-PSPAAALPPRSAFLPRFALPITSTTQKRHRL
Borage AHAS1 (1) ----MASTPPSSTLTHPT-TTPSSFPNHPKLFSSSFTLPFPVSPQTTSLS
Borage AHAS2 (1) ----MTATPHSSTLTHPT-PTPTSFPSHPKLFSSSFTLPFPLSPQTTSLS
Borage AHAS1 mutant (1) ----MASTPPSSTLTHPT-TTPSSFPNHPKLFSSSFTLPFPVSPQTTSLS
Borage AHAS2 mutant (1) ----MTATPHSSTLTHPT-PTPTSFPSHPKLFSSSFTLPFPLSPQTTSLS
51 100
A thaliana AHAS (49) SRRRGIKSSSPSSISAVLNTTTTN--VTTTPSPTKPTKPETFISRFAPDQP
Sunflower AHAS1 (45) H-----------ISNVLSDSKS----TTTTTQPPLQAQPFVSRYAPDQP
Borage AHAS1 (46) HSKH-LRRHSLHPISNVISTRPSTSSPSSQNTPEQKEQLPFISRYAPNEP
Borage AHAS2 (46) HTKH-IRRNSLHPISNVISPSP---IPSSQSTPQQK-QPPFISRYAPEEP
Borage AHAS1 mutant (46) HSKH-LRRHSLHPISNVISTRPSTSSPSSQNTPEQKEQLPFISRYAPNEP
Borage AHAS2 mutant (46) HTKH-IRRNSLHPISNVISPSP---IPSSQSTPQQK-QPPFISRYAPEEP
101 150
A thaliana AHAS (97) RKGADILVEALERQGVETVFAYPGGASMEIHQALTRSSSIRNVLPRHEQG
Sunflower AHAS1 (79) RKGADVLVEALEREGVTDVFAYPGGASMEIHQALTRSNTIRNVLPRHEQG
Borage AHAS1 (95) RKGADVLVEALERQGVTNVFAYPGGASMEIHQALTRSNIIKNVLPRHEQG
Borage AHAS2 (91) RKGADVLVEALEREGVTNVFAYPGGASMEIHQALTRSNIIKNVLPRHEQG
Borage AHAS1 mutant (95) RKGADVLVEALERQGVTNVFAYPGGASMEIHQALTRSNIIKNVLPRHEQG
Borage AHAS2 mutant (91) RKGADVLVEALEREGVTNVFAYPGGASMEIHQALTRSNIIKNVLPRHEQG
151 200
A thaliana AHAS (147) GVFAAEGYARSSGKPGICIATSGPGATNLVSGLADALLDSVPLVAITGQV
Sunflower AHAS1 (129) GVFAAEGYARASGLPGVCIATSGPGATNLVSGLADALLDSVPMVAITGQV
Borage AHAS1 (145) GVFAAEGYARASGEPGVCIATSGPGATNLVSGLADALLDSVPMVAITGQV
Borage AHAS2 (141) GVFAAEGYARASGDPGVCIATSGPGATNLVSGLADALLDSVPMVAITGQV
Borage AHAS1 mutant (145) GVFAAEGYARASGEPGVCIATSGPGATNLVSGLADALLDSVPMVAITGQV
Borage AHAS2 mutant (141) GVFAAEGYARASGDPGVCIATSGPGATNLVSGLADALLDSVPMVAITGQV
201 250
A thaliana AHAS (197) PRRMIGTDAFQETPIVEVTRSITKHNYLVMDVEDIPRIIEEAFFLATSGR
Sunflower AHAS1 (179) PRRMIGTDVFQETPIVEVTRSITKHNYLVLDVEDIPRIVREAFYLASSGR
Borage AHAS1 (195) PRRMIGTDAFQETPIVEVTRSITKHNYLVLNVDDIPRIVKEAFYLARSGR
Borage AHAS2 (191) PRRMIGTDAFQETPIVEVTRSITKHNYLVLSVDDIPRIVKEAFYLARSGR
Borage AHAS1 mutant (195) PRRMIGTDAFQETPIVEVTRSITKHNYLVLNVDDIPRIVKEAFYLARSGR
Borage AHAS2 mutant (191) PRRMIGTDAFQETPIVEVTRSITKHNYLVLSVDDIPRIVKEAFYLARSGR
251 300
A thaliana AHAS (247) PGPVLVDVPKDIQQQLAIPNWEQAMRLPGYMSRMPKPPEDSHLEQIVRLI
Sunflower AHAS1 (229) PGPVLIDVPKDIQQQLVVPKWDEPMRLPGYLSRMPKPQYDGHLEQIVRLV
Borage AHAS1 (245) PGPVLIDVPKDIQQQNVVPNWDVEMGLCGYISRLCKPPSELLLEQIVRLI
Borage AHAS2 (241) PGPVLIDVPKDIQQQMVVPHWDVEMGLSGYISRLCKPPCELLLEQIVRLI
Borage AHAS1 mutant (245) PGPVLIDVPKDIQQQNVVPNWDVEMGLCGYISRLCKPPSELLLEQIVRLI
Borage AHAS2 mutant (241) PGPVLIDVPKDIQQQMVVPHWDVEMGLSGYISRLCKPPCELLLEQIVRLI
301 350
A thaliana AHAS (297) SESKKPVLYVGGGCLNSSDELGRFVELTGIPVASTLMGLGSYPCDDELSL
Sunflower AHAS1 (279) GEAKRPVLYVGGGCLNSDDELRRFVELTGIPVASTLMGLGAYPASSDLSL
Borage AHAS1 (295) SEAKKPVLYVGGGCLNSSEELKRFVELTGIPVASTLMGLGSFPGSDELSL
Borage AHAS2 (291) SEAKRPVLYVGGGCLNSSEELKRFVELTGIPVASTLMGLGSFPGSDELSL
Borage AHAS1 mutant (295) SEAKKPVLYVGGGCLNSSEELKRFVELTGIPVASTLMGLGSFPGSDELSL
Borage AHAS2 mutant (291) SEAKRPVLYVGGGCLNSSEELKRFVELTGIPVASTLMGLGSFPGSDELSL
351 400
A thaliana AHAS (347) HMLGMHGTVYANYAVEHSDLLLAFGVRFDDRVTGKLEAFASRAKIVHIDI
Sunflower AHAS1 (329) HMLGMHGTVYANYAVDKSDLLLAFGVRFDDRVTGKLEAFASRAKIVHIDI
Borage AHAS1 (345) QMLGMHGTVYANYAVDKSDLMLAFGVRFDDRVTGKLEAFASRAKIVHIDI
Borage AHAS2 (341) QMLGMHGTVYANYAVDKSDLMLAFGVRFDDRVTGKLEAFASRAKIVHIDI
Borage AHAS1 mutant (345) QMLGMHGTVYANYAVDKSDLMLAFGVRFDDRVTGKLEAFASRAKIVHIDI
Borage AHAS2 mutant (341) QMLGMHGTVYANYAVDKSDLMLAFGVRFDDRVTGKLEAFASRAKIVHIDI

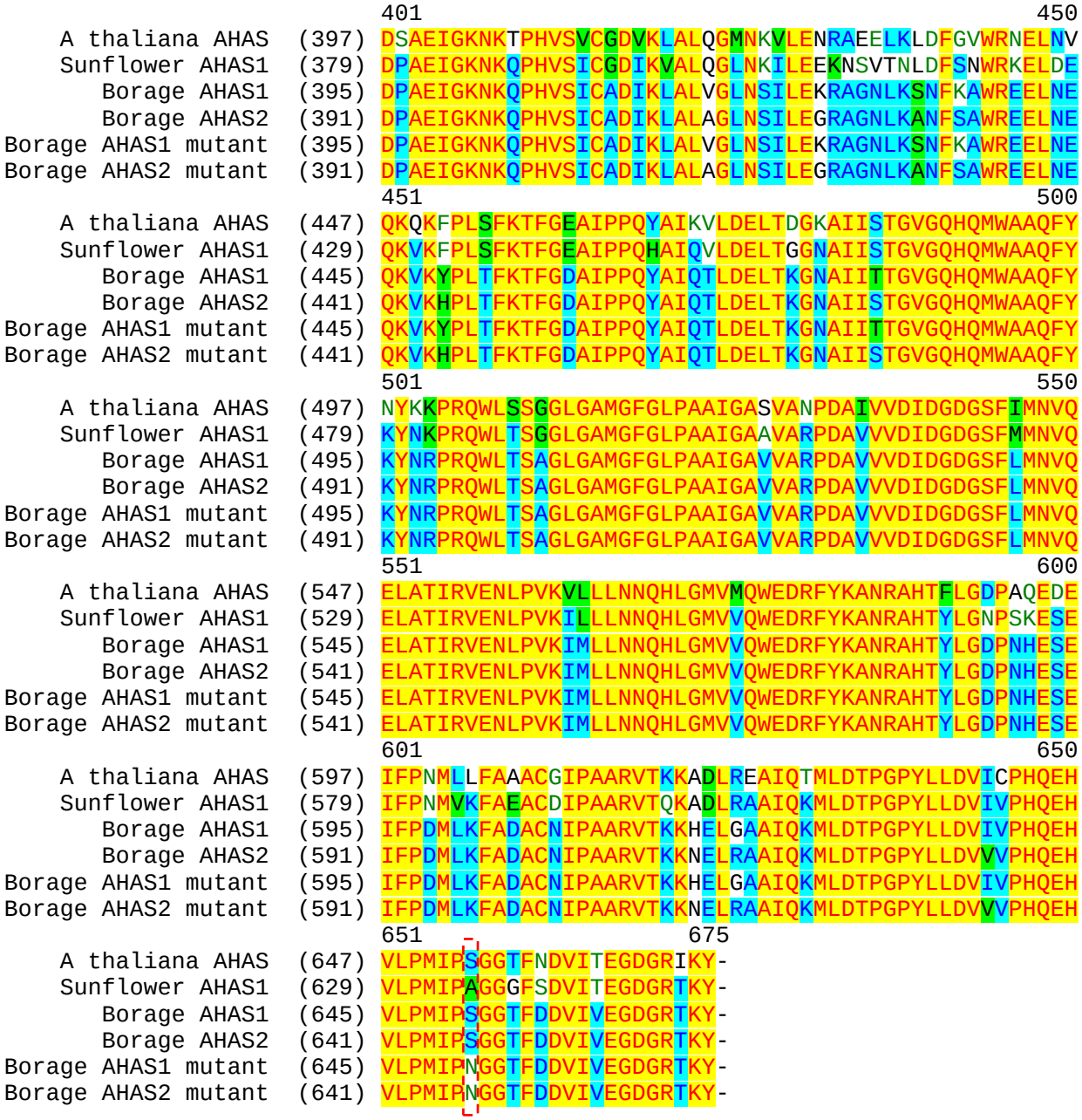


Figure 16. Alignment of AHAS protein sequences from *Arabidopsis thaliana*, sunflower (*Helianthus annuus*) and both susceptible and resistant borage lines. The completely and partially identical amino acids were highlighted in yellow and blue color, respectively. Amino acid substitutions in *AHAS1* and *AHAS2* of two separate tolerant lines were highlighted in the red dashed box.

4.2.6 Discussion

Two AHAS genes were isolated in borage. They showed very high homology with each other, up to 95% identity at the amino acid level. Both AHAS protein sequences also share greater than 75% identity with Arabidopsis AHAS and sunflower AHAS. AHAS genes have been identified and sequenced in a variety of plants, fungi, algae and bacteria. The similarity of AHAS protein sequences among different species ranges from 17% to 90%. Many residues of AHAS enzymes are absolutely conserved across species. In most plant species, at least one AHAS gene is expressed in a constitutive manner, as *AHAS* is known as housekeeping gene (Duggleby and Pang, 2000). Some plants, such as *N. tabacum, B. napus* and *G. hirsutum*, contain more than one AHAS genes. The presence of multiple AHAS genes may be derived from a polyploidy process by the combination of genomes of their diploid progenitors (Lee *et al*., 1988; Grula *et al*., 1995; Rutledge *et al*., 1991). In some plants, there are two housekeeping AHAS genes expressed at about same level. In *B. napus* and *G. hirsutum,* there is another AHAS gene expressed in tissue specific manner (Duggleby and Pang, 2000). Interestingly, *B. napus* also contains a fourth AHAS gene which is considered as a pseudogene and not expressed (Ouellet *et al.,* 1992). This study revealed that borage has at least two AHAS genes that are constitutively expressed as both cDNAs were retrieved from the leaf tissue. However, their real expression pattern would require the examination of their expression levels in different tissues using real-time qRT-PCR.

Sequence comparison identified a point mutation in the coding region of *AHAS1* and *AHAS2* respectively in two different imidazolinone resistant borage plants. The point mutation results in an amino acid change from serine to asparagine in the AHAS proteins (Figure 16). Previous studies showed the most common mutations for herbicide resistance are at residues

A122, P197, A205, W574 and S653 referring to Arabidopsis AHAS protein sequence (Duggleby and Pang, 2000). A mutation at S653 was first discovered by Hattori *et al*. (1992) in imidazolinone resistant Arabidopsis. According to the early research (Duggleby *et al.*, 2008; Lee *et al*., 2011), the amino acid substitution resides in the γ-domain at the C-terminal end of the catalytic subunit of AHAS enzymes. The catalytic subunit aggregates to form a tetramer complex with another tetramer of four regulatory subunits to constitute the AHAS apoenzyme. The serine residue in the position of AHAS enzymes is relatively conserved across species, although an alanine in the cocklebur enzyme, a glycine in the yeast enzyme and in *E. coli* AHAS III and a proline in *E. coli* AHAS I and II are also observed (Bernasconi *et al*., 1995; Sathasivan *et al*., 1991). In addition to resistance to imidazolinones, the mutation at this site is characterized by cross-resistance to pyrimidyl oxybenzoates, but not to sulfonylureas and triazolopyrimidines (Mourad and King, 1992; Sathasivan *et al*., 1991). Using *in vitro* mutagenesis, the serine residue was mutated to different amino acids such as alanine, threonine and phenylalanine. The alanine substitution is sensitive to sulfonylureas and imidazolinones, while S653T, S653N and S653F mutations result in enzymes with 10 fold or more resistance to imidazolinones (Duggleby and Pang, 2000).

4.3 Study 3: Development of KASP SNP marker linked to imidazolinone resistance gene (*AHAS1* mutation) in borage

4.3.1 Abstract

Based on the single nucleotide substitution of the AHAS1 gene in the herbicide resistant borage, a set of primers was designed to develop the KASP SNP marker. The result showed that the marker could readily distinguish wild type, heterozygous and homozygous plants. This robust

and user-friendly KASP SNP marker would be very useful for routine marker-assisted selection of imidazolinone resistant borage.

4.3.2 Hypothesis

KASP is an inexpensive, versatile and sensitive SNP genotyping system to detect single nucleotide mutation, thus it can be used to differentiate homozygous herbicide-resistant, homozygous herbicide-susceptible and heterozygous herbicide-resistant borage genotypes.

4.3.3 Introduction

The herbicide resistant mutation is generally dominant; therefore genotypes of homozygous and heterozygous tolerant plants are difficult to distinguish from phenotypes. However, since imizadolinone resistance of borage is induced by a single nucleotide substitution, the SNP can be detected by KASP technology and used as a marker to distinguish the genotypes. The KASP genotyping system is an accurate and cost-effective fluorescence-based technology developed by KBioscience for high-throughput SNPs genotyping (Semagn *et al*. 2014). The technology is based on allele-specific oligo extension and fluorescence resonance energy transfer (FRET) for signal generation (Kumpatla *et al*. 2012). Two allele-specific primers and one allele common primer are included in a KASP genotyping assay. Each allele-specific primer must be ended with a specific nucleotide of the SNP and attached with unique unlabeled tail sequences at the 5’ end. The mixture of FAM and HEX specific FRET cassette in the master mix will bind the unique target tail sequences to produce fluorescence with either only one or mixed type of the signals (LGC Genomics, 2014). According to the signal generated, sample materials can be assigned to homozygous and heterozygous genotypes. This study aims to use the KASP technology to genotype the herbicide resistant borage plants.

4.3.4 Experimental approach

Forty M3 borage plants from a single *AHAS1* mutant line were randomly selected and numbered. Their leaf tissues were collected at 2 - 4 leaf stage. Leaf samples were snap-frozen in liquid nitrogen and stored at -80°C for genomic DNA extraction. Forty plants were kept for imidazolinone screening to validate the result of KASP genotyping. Genomic DNA was extracted by adapting CTAB method (Schnable Lab, 2014) and DNA concentration was diluted to 5 ng/μL. DNA samples were stored at -20°C for further use. A set of primers (Table 6) was designed and synthesized for KASP genotyping following the manual from LGC genomics. Each set of primers consists of two gene-specific primers and one common primer. Gene-specific primers contain a unique unlabeled tail sequence at the 5' end. Gene-specific primers have to end with the SNP at the 3' end. In this case, two specific primers are reverse primers and the common primer is forward.

Table 6. Primers for KAPS genotyping (Red color highlighted sequence in primer 1 is unlabeled oligo sequence and primer 1 ends with mutant nucleotide "T"; blue color highlighted sequence in primer 2 is unlabled oligo sequence and primer 2 ends with original nucleotide "C").

Primer	Sequence
Reverse Allele-Specific Primer 1	GAAGGTCGGAGTCAACGGATTGATAACATCATCAAAGGTTCCGCCAT
Reverse Allele-Specific Primer 2	GAAGGTGACCAAGTTCATGCTATAACATCATCAAAGGTTCCGCCAC
Forward Common Primer	CGGACCATACTTATTGGATGTCATTGTC

KASP genotyping assay was performed on StepOne Real-time PCR system (Applied Bioisystems). On a 96-well plate, each well contained about 10 μL of reaction mixture, including

5 μL of DNA sample, 5 μL of 2X master mix (LGC genomics), 0.14 μL of primer mix. Four homozygous susceptible controls and two no-template controls were also included. KASP assay thermal cycling program was as follows: pre-read at 25°C for 30s; holding at 95°C for 15s; 10 cycles X (95°C for 20s, 61°C for 60s); 30 cycles X (95°C for 20s, 55°C for 60s); post-read at 25°C for 30s.

4.3.5 Results

The individual genotype of a segregating population of forty M3 borage samples was determined by KASP genotyping PCR. The allelic discrimination plot based on the PCR result was shown in Figure 17 and the segregation result of 40 borage samples based on KASP genotyping was summarized in Table 7. As shown in the figure, the plant genotypes could be divided into three groups. The homozygous herbicide resistant plants represented by homozygous FAM (fluorescein amidite) allele marked by red were clustered in the lower right corner of the plot, the homozygous susceptible plants represented by the homozygous HEX (5-hexadecanoyl fluorescein) allele marked by blue were clustered in the upper left corner of the plot, while the heterozygous plants marked by green are clustered in the central region of the plot. It was noted that the KASP genotypes of individuals was consistent with their herbicide spraying phenotype and overall ratio of herbicide tolerant plants to susceptible plants was 31:9, equivalent to the theoretical ratio 3:1 [X^2 (1, N = 40) = 0.133, p < 0.01] (Table 7).

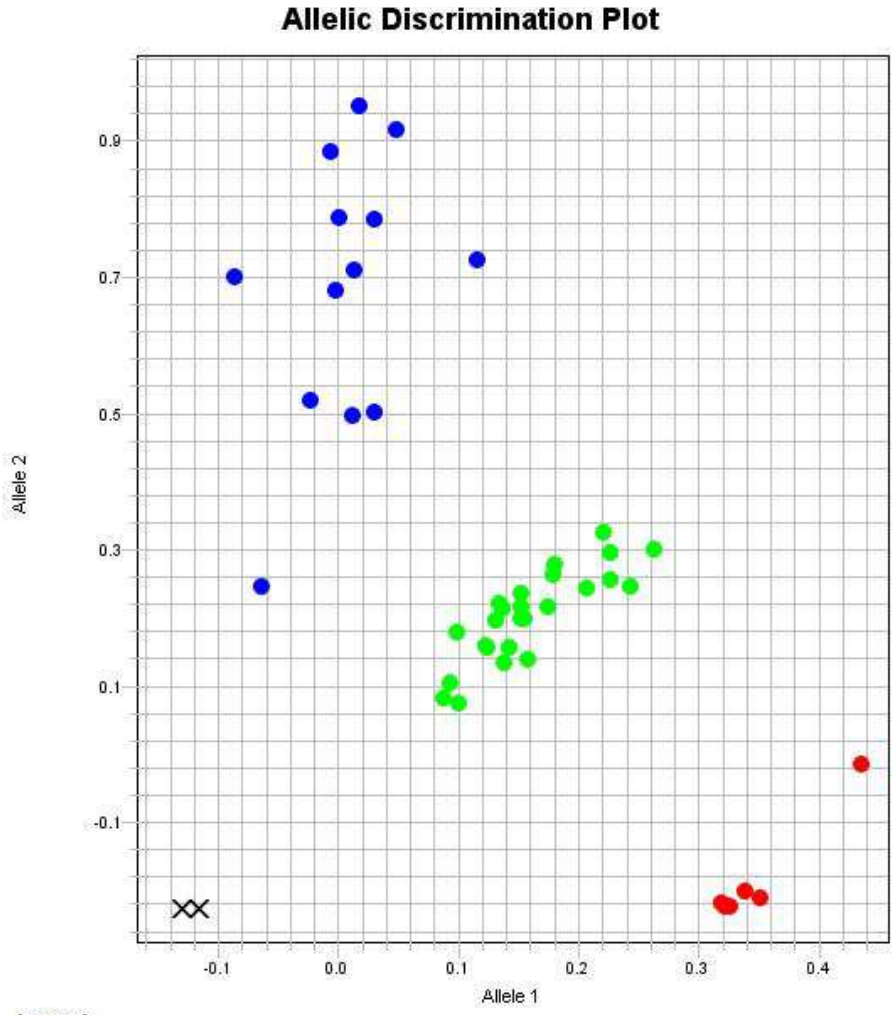


Figure 17. KASP genotyping plot for M3 borage plants. Samples marked red are the homozygous resistant for the FAM allele, blue are the homozygous susceptible for the HEX allele and green are the heterozygous; "X" are two no-template controls.

Table 7. Segregation of M3 borage plants based on the KASP genotyping result. The homozygous resistant, RR; the homozygous susceptible, rr; the heterozygous, Rr.

Segregation of M3 Borage Plants		
Resistant (RR)	Susceptible (rr)	Heterozygous (Rr)
6	9	25

4.3.6 Discussion

In this study, the KASP assay was successfully used to identify and differentiate the homozygous resistant, homozygous susceptible and heterozygous plants in the segregation population. The result was confirmed by phenotyping of the herbicide resistance. The herbicide screening showed among the 40 plants, 9 of them were susceptible and 31 were tolerant, which was consistent with those identified by the KASP genotyping result. As described in the previous study (Section 4.1), the M3 borage population was expected to segregate into imidazolinone resistant and susceptible groups by 3:1 ratio. The number of the resistant to the susceptible at 31:9 from the KASP genotyping (Table 7) is close to the expected ratio. In addition, imidazolinone susceptible controls and no-template controls are readily distinguished in the assay, indicating that the sensitivity of KASP assay is excellent. Therefore, the KASP assay is being used to select imidazolinone resistant traits for rapid genotyping and seed increase in our breeding program. After the KASP genotyping and herbicide-screening phenotyping validation, approximately 20 homozygous resistant plants of the M4 generation were determined and selected for the breeding. It is noteworthy that the KASP marker for the imidazolinone-resistant SNP of AHAS2 gene can also be developed following the same procedure. However, due to time limitation, this is not pursued further in this study.

4.4 Study 4: *In vitro* AHAS activity assays of the resistant and susceptible borage plants

4.4.1 Abstract

Crude proteins from leaf tissues of both susceptible (wild-type) and resistant (*AHAS1* mutant) plants were extracted, and used as enzyme sources for the AHAS activity assay. The protein extract containing the AHAS enzymes was incubated with the substrate and cofactors in

a buffer with or without imazamox, the herbicide active ingredient. The catalytic reaction transformed the substrate to acetolactate that was then further converted to acetoin. The detection of acetoin via the formation of a creatine and naphthol complex was used to determine the AHAS activity of susceptible and resistant plants.

4.4.2 Hypothesis

Herbicide imazamox is an inhibitor of the AHAS enzyme. Homozygous herbicide-resistant borage line (*AHAS1* mutant) should retain significantly higher AHAS activity than the herbicide-susceptible borage (wild-type) when assayed in presence of the inhibitor.

4.4.3 Introduction

It has been shown from the above studies that the *AHAS1* mutant borage line can tolerate two times the recommended dosage of "Solo" herbicide and the tolerance is caused by the single nucleotide mutation of the AHAS1 gene. As such, the AHAS activity level of the mutant plants would provide further evidence to support the conclusion. The AHAS enzyme activity is generally measured using a discontinuous colorimetric assay based on the method developed by Singh *et al.* (1988). In this method, the crude protein is incubated with the substrate for a fixed time to generate intermediate acetolactate that is then converted to acetoin under decarboxylation. Finally, the reaction of acetoin with creatine and α-naphthol forms a pink-colored product which can be measured at 520 nm wavelength in a spectrometer. As a result, the AHAS activity level can be estimated based on the color density (Duggleby and Pang, 2000; Singh *et al*., 1988; Westerfeld, 1945). This study aims to determine AHAS activity levels of the resistant and susceptible borage plants using in vitro AHAS assays.

4.4.4 Experimental approach

4.4.4.1 Preparation of enzyme sources

In vitro assay of AHAS activity for each imazamox concentration (0, 1, 5, 25, 125, 625 μM) was performed with three biological samples per genotype, and each biological sample consisted of two technical replicates. At the 4 - 6 leaf stage, the leaf material (about 3 - 4 g) was harvested and snap-frozen in liquid nitrogen and stored at -80°C. The *in vitro* assay was conducted according to the method of Yu *et al*. (2004) with modifications. About 1 g of the frozen material was ground to fine powders with a mortar and pestle in liquid nitrogen, and homogenized in 4 volumes of cold extraction buffer containing 0.1 M K_2HPO_4 (pH 7.5), 10 mM sodium pyruvate, 0.5 mM $MgCl_2$, 0.5 mM thiamine pyrophosphate (TPP), 10uM flavin adenine dinucleotide (FAD), 4 mM DTT, 1 mM phenylmethylsulphonyl fluoride (PMSF), 10% v/v glycerol, and 4% soluble PVP. The homogenate was filtered through two layers of miracloth and the filtrate was centrifuged at 30000 g for 20 minutes at 4°C. The supernatant was firstly brought to 30% saturation by drop-wise addition of solid $(NH_4)_2SO_4$ to allow unknown "gums" to form and to be removed; the supernatant was then brought to 50% saturation with $(NH_4)_2SO_4$. The solution was allowed to stand on ice for 10 min with occasional stirring and any additional "gums" would be removed or filtered out. The sample solution was then divided into two as technical replicates before going to centrifugation. After centrifugation at 100,000 g for 20 minutes at 4°C, the gummy protein layer was carefully collected as enzyme sources for the activity assay.

4.4.4.2 Enzyme incubation and colorimetric reaction

The gummy protein layer was re-dissolved in 1.4 mL incubation buffer containing 50 mM K_2HPO_4 buffer (pH 7.0), 100 mM sodium pyruvate, 10 mM $MgCl_2$, 1 mM TPP and 1 μM FAD. The amount of proteins in each sample was determined immediately by the Bio-Rad

protein assay using a dye reagent (#500-0006). A series of concentrations at 0, 1, 5, 25, 125, 625 μM imazamox PESTANAL®, analytical standard (Sigma-Aldrich Co. LLC) were added to 200 μL of the reaction mixture, respectively. The mixture was incubated at 37°C for 1 hour. To stop the reaction, 32 μL of 1 M H_2SO_4 was added, and decarboxylation occurred at 65°C for 15 minutes. Then the sample was incubated with 34 μL of creatine solution (1% w/v in 2N NaOH) and 68 μL of α-napthol solution (5% w/v in 2N NaOH) at 60°C for 15 minutes. After cooled down for 10 minutes at the room temperature to maximize the color development, the mixture was briefly centrifuged at 13000g. 200 μL of the reaction solution was transferred to a 96 well microtiter plate for measurement of the absorbance at 520nm. The background control for non-AHAS activity was determined by adding 32 μL of 5N NaOH to 200 μL of the reaction mixture after 1 hour of incubation.

The unit of enzyme activity was defined as micromole of acetoin produced, and specific activity of AHAS enzyme in resistant and susceptible borage was calculated on the basis of micromole acetoin produced per millgram of the protein and per minute of the reaction time. Therefore, in order to quantify enzymatic levels, standard curves of acetoin were generated using a series of acetoin dilutions in the incubation buffer.

4.4.5 Results

The *in vitro* AHAS activity assay was based on measurement of acetoin produced by the AHAS enzyme in presence of the substrate. The production of acetoin shown by pink color products was measured by the colorimetric absorbance reflecting the activity of AHAS enzyme. The higher the activity, the stronger the pinkness. The visual inspection of the assay (Figure 18) showed intensity of the pink color of the acetoin complex produced in both the mutant and wild-type were gradually reduced when imazamox concentrations were increasing in the assays.

However the pinkness of the resistant line remained stronger than that of the susceptible line, especially at high concentrations of imazamox from 25 μM to 625 μM. This result indicated that although increased inhibition of the total AHAS enzyme activity occurred with increased concentration of imazamox in both borage lines, the imidazolinone resistant mutant line was able to retain significantly greater AHAS activity than the susceptible wild type in the range of imazamox concentrations.

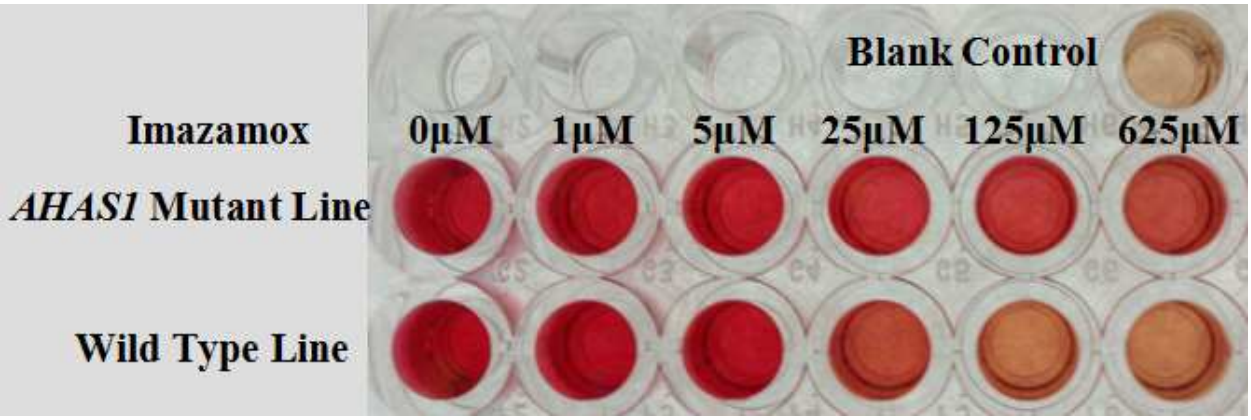


Figure 18. *In vitro* colorimetric assays of AHAS activity of the *AHAS1* mutant and wild type borage plants in a range of imazamox concentrations.

Quantitative result of specific AHAS activity across a range of imazamox concentration between the two genotypes was shown in Figure 19. Statistical analysis of the data using Factorial Treatment Arrangement on CRD (completely randomized design) (Appendix 3) indicated that the mutant borage had significantly higher AHAS activity than the wild type borage across all imazamox concentrations, although the specific AHAS activities in both lines were gradually decreased with the imazamox concentrations increased. The activity of the mutant line could retain up to 20% of the total activity at zero μM of imazamox while that of susceptible borage went down to zero at 625 μM of imazamox.

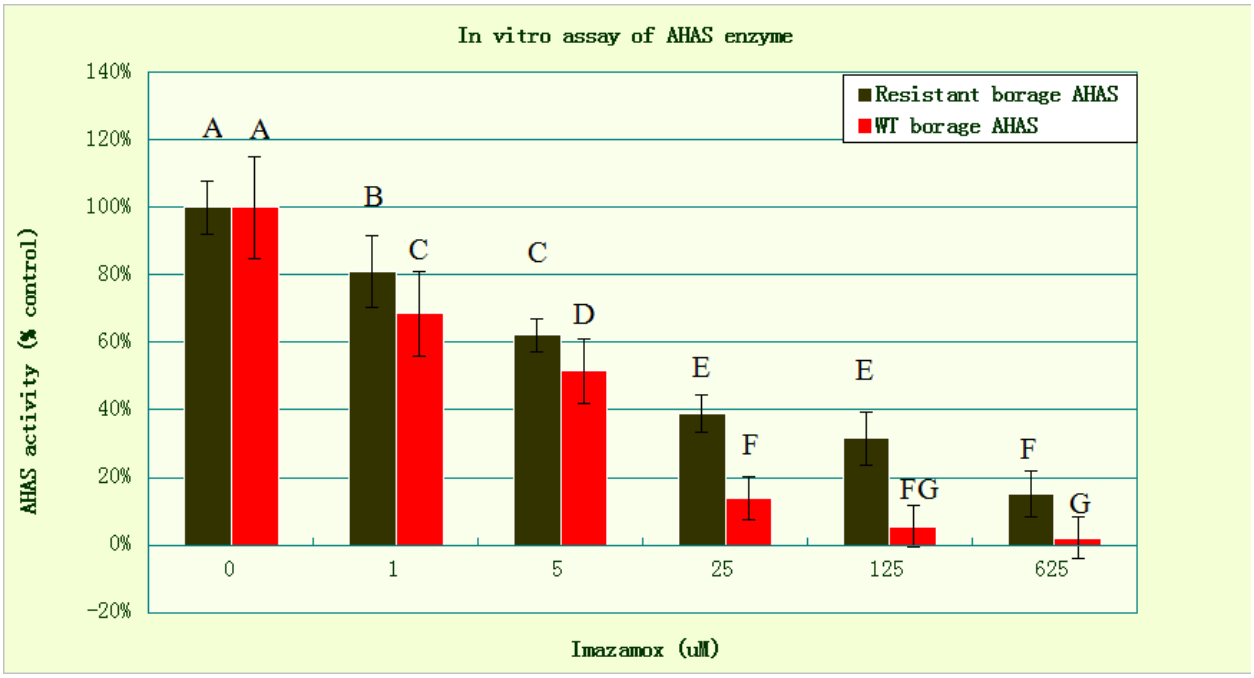


Figure 19. Comparison of specific AHAS activities between the *AHAS1* mutant and wild type across different imazamox concentrations. The activity at 0 μM imazamox was as 100%; the same letter means that the activities are not significantly different (P >0.05).

4.4.6 Discussion

In the AHAS activity assay, a background control representing acetoin production by non-AHAS activity was included, as a number of acetoin-forming enzymes including pyruvate decarboxylase (PDC) in plant tissues might interfere with the assay (Forlani *et al.*, 1999; Muhitch, 1988). For instance, a previous study showed that non-AHAS activity in shoot tissue of *L. Rigidum* plant could account for 15 - 30% of the total acetoin production. These non-AHAS enzymes catalyze formation of acetoin via non-acidic conversion (Yu *et al.*, 2010), which could be estimated using NaOH instead of H_2SO_4 to terminate the reaction (Pornprom *et al.*, 2005; Tanaka, 2003). In addition, non-AHAS enzymes such as PDC have been shown to reduce the sensitivity of AHAS enzymes to herbicide or feedback inhibition of branched chain amino acids

in maize kernels (Muhitch, 1988). The borage AHAS assay in this study showed that acetoin produced by non-AHAS enzymes in leaf tissues accounts for approximately 28% of the total acetoin production (data not shown) and this part of acetoin production was thus excluded from the AHAS activity (Figure 19). As seen in Figure 19, *in vitro* AHAS activity of imidazolinone resistant borage was significantly higher than susceptible borage across all imazamox concentrations tested. The activity of the resistant line could retain up to 20% of the total activity while that of susceptible borage went down to zero at 625 μM imazamox. This result is in agreement with the early research that S653N mutation of an AHAS gene could confer strong tolerance to imidazolinones (Duggleby and Pang, 2000). The serine residue at position 653 located at substrate binding channel is critical for interaction with a substrate, and substitution of the amino acid to asparagine would prevent the herbicide from binding to AHAS, resulting in insensitivity and tolerance of the enzyme to imidazolinones (Section 3.5). One of another possible explanation for higher AHAS activity of the resistant line is that the mutant AHAS gene confers increased enzymatic stability (Yu *et al*., 2010) as wild type plant AHAS is more sensitive to the operational procedure of enzyme extraction and purification (Duggleby and Pang, 2000; Muhitch, 1988; Yu *et al*, 2003; 2007*a, b*; 2010). In addition, the higher activity of AHAS in the mutant line may be due to improved ability of cofactor binding or improved stability in the catalytic subunit when interacting with the regulatory subunit of AHAS enzyme (Chang and Duggleby, 1998; Kim *et al*., 2004; Yu *et al*, 2010).

Although imidazolinone resistant borage showed significantly higher AHAS activity compared to the wild type in the assay, the overall enzyme activity in presence of imazamox was gradually decreased with the concentrations increased. As discussed above, borage has two AHAS genes in the genome that are co-expressed in leaf tissues. If one AHAS gene is mutated in

the resistant line (*AHAS1),* the other (*AHAS2)* would remain intact. As a result, the total AHAS activity would be reduced even in the *AHAS1* mutant line as AHAS2 enzyme is inhibited by the herbicide. However, with the concentration of the imazamox increasing to a very high level, such as 625 μM, the mutant line also becomes less tolerant to the herbicide, resulting in approximately 20% of the total AHAS activity, while the AHAS activity of the susceptible line is completely inhibited by such level of herbicide (Figure 18 and Figure 19). There are reports that *in vitro* AHAS activity is only slightly reduced or unchanged in resistant lines of some dicot plant species (Ashigh and Tardif, 2007; Boutsalis *et al*., 1999; Eberlein *et al*., 1997, 1999; Preston *et al.,* 2006). The resistance difference between *in vitro* AHAS activity and the whole-plant performance can thus be related to the number of AHAS genes in a plant species, and the location of a mutation in a AHAS gene (Yu *et al.,* 2010) as well as the binding affinity of a herbicide and the toxic strength of the herbicide to the target enzyme (Ashigh and Tardif, 2007).

Borage contains a high level of polyphenols in leaf tissues. These polyphenolic compounds may inhibit enzyme activity directly or indirectly by hydrogen bonding with peptide bond oxygens or by covalent modification of amino acid residues (Gegenheimer, 1990). Therefore, in order to remove or inactivate the polyphenols, polyvinylpyrrolidone (PVP) or polyvinylpolypyrolidone (PVPP) and dithiothreitol (DDT) was included in the enzyme extraction buffer to reduce polyphenol interference and maintain a strong reducing environment to counteract the effect of phenol oxidases (Gegenheimer, 1990). Nevertheless, an unknown yellow gummy layer was still observed after precipitation of the enzymatic extract which appeared to be able to bind, adhere or absorb the AHAS enzyme. Although it did not affect the AHAS activity in a very dramatic way, it would be interesting to know what the chemical nature of the gummy substance is and how this compound can be removed from the enzyme.

4.5 Study 5: Herbicide type and dosage responses of the *AHAS1* mutant borage line

4.5.1 Abstract

A series of concentrations of a "Solo" herbicide was applied to the borage plants of the mutant line (*AHAS1*) in a greenhouse to test the resistant level. The result indicated that the mutant borage was tolerant to four times the field applied concentration of the "Solo" herbicide. In addition, different types of the group 2 herbicides were also tested to determine whether the *AHAS1* mutation would confer any cross-resistance among the group 2 herbicides. The result showed that the *AHAS1* mutation exhibited strong resistance to both imazethapyr and imazamox herbicides as well as some tolerance to flucarbazone herbicide.

4.5.2 Hypothesis

The mutant borage plants were selected from tolerance to imazamox, the active ingredient of an imidazolinone herbicide. It is possible that it can also be tolerant to other imidazolinone herbicides within the group with the dosage response.

4.5.3 Introduction

The *AHAS1* mutant borage line with the single amino acid substitution (S653N) was obtained by screening an EMS mutagenized population using two times the recommended dosage of "Solo" herbicide. According to Duggleby and Pang (2000), the mutation S653N of AHAS gene results in *Arabidopsis thaliana* with resistance of 100 fold or more to imidazolinones. In addition, other studies showed that the S653 mutation confers tolerance only to imidazolinones, but not to other chemical families in group 2 herbicides (Dietrich, 1998; Lee *et al.*, 1999; Sathasivan *et al.*, 1991; Tan *et al.* 2006; Tranel and Wright, 2002; Tranel *et al.*, 2003). This study aims to determine the type and level of herbicide resistance conferred by the *AHAS1* mutant line.

4.5.4 Experimental approach

4.5.4.1 Herbicide dosage response test

Herbicide response tests were carried out in a greenhouse in the Innovation Place (Saskatoon). M4 homozygous imidazolinone-resistant borage (*AHAS1* mutant line) were planted at 1 - 2 cm in 25 x 50 cm flats containing commercial potting mix (Sunshine Mix 3; Sun Gro.) in the growth chamber under a 16 hour light (22°C) and 8 hour dark (16°C) cycle. Each flat contained 36 seeds. A group 2 herbicide, “Solo”, was applied over foliage when most plants were at two-leaf stage in an herbicide chamber. The spray solutions included 2X (84g ai/ha imazamox), 4X, 8X, 16X, 32X, 64X, 128X and 256X of “Solo” with adjuvant Merge at 0.5% (v/v) of the solution volume. A moving nozzle cabinet sprayer with a flat-fan nozzle tip was calibrated to deliver 102 L/ha of the spray solution in a single pass. Sprayed M4 plants were visually evaluated at 21 days after imazamox application by comparing with untreated controls.

4.5.4.2 Herbicide type response test

By following a similar procedure above, M4 homozygous imidazolinone-resistant borage (*AHAS1* mutant) was tested with 8 types of group 2 herbicides including Solo (84g ai/ha imazamox, BASF), Muster (45g ai/ha ethametsulfuron-methyl, DuPont), Pursuit (102g ai/ha imazethapyr, BASF), Everest 2.0 (116g ai/ha flucarbazone, Arysta LifeScience), PrePass XC (20g ai/ha florasulam, Dow AgroScience), Pinnacle SG (11g ai/ha thifensulfuron, DuPont), Express SG (32g ai/ha tribenuron methyl, DuPont) and Accent (51g ai/ha nicosulfuron, DuPont) at 2X of the recommended rate. Adjuvant reagent for each herbicide was added into spray solution accordingly. Sprayed M4 borage plants were visually evaluated at 21 days after spraying by comparing with wild-type controls.

4.5.5 Results

The herbicide dosage response test showed that 100% of the survival rate without any obvious injury was observed after four times “Solo” herbicide treatment (Figure 20). With the concentration increasing to eight times, the mutant plants showed injury symptoms. However, all of the wild type plants were completely wiped out by the herbicide at 2X concentrations (Figure 20). This result indicated the mutant borage (*AHAS1*) was tolerant up to four times the recommended dosage of imidazolinone herbicides, whereas all wild type plants were not tolerant to the treatment.

Figure 20. Herbicide dosage response test showed that homozygous resistant borage tolerated up to 4X “Solo” herbicide. Tray 1 was wild-type borage control without herbicide treatment; tray 2

was homozygous resistant borage treated with 2X “Solo”; tray 3 was homozygous resistant borage treated with 4X “Solo”; tray 4 was wild-type borage control treated with 2X “Solo”. The image was taken at 21 days after the treatment.

Testing of different herbicides within Group 2 showed that besides imazamox, the *AHAS1* mutant line was also highly tolerant to “Pursuit” (imazethapyr) with no obvious chemical damage (Figure 21). Interestingly, the *AHAS1* mutant line also showed moderate tolerance to “Everest 2.0” herbicide (flucarbazone sodium) (Figure 21). Other than that, the mutant line was sensitive to the other group 2 herbicides tested (Table 8).

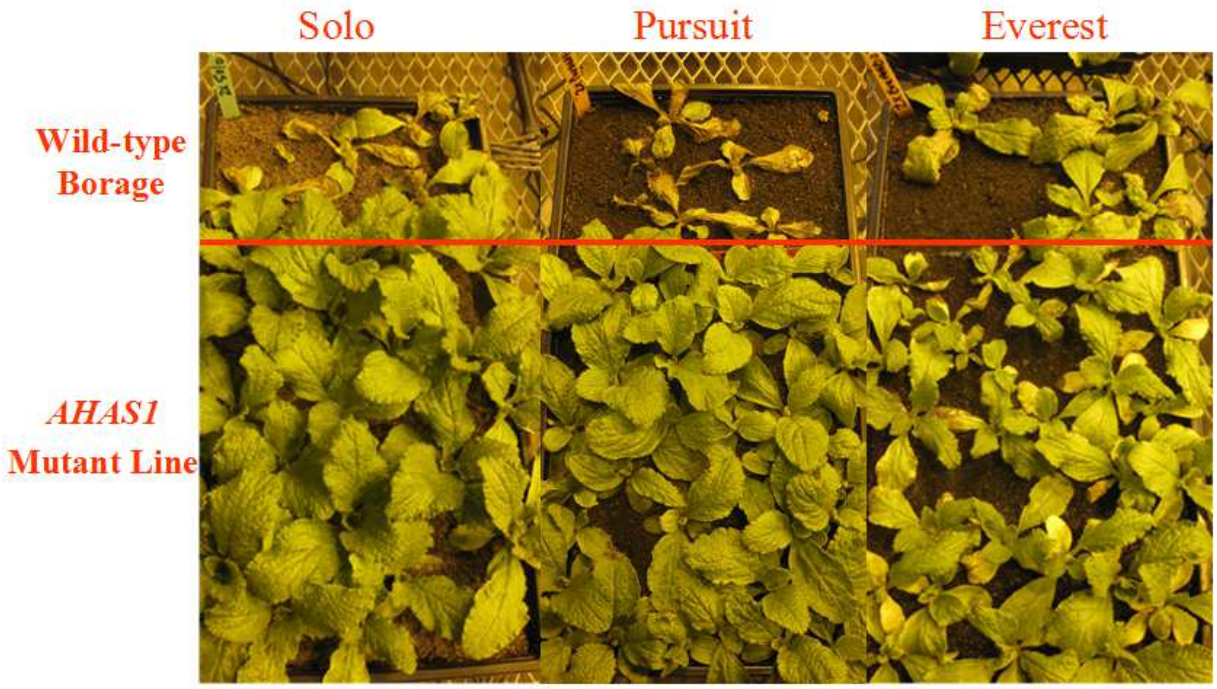


Figure 21. The M4 homozygous resistant borage showed strong resistance to herbicide “Solo” and “Pursuit”, and it also exhibited moderate tolerance towards “Everest 2.0”. From left to right: The mutant borage treated with 2X “Solo”; The mutant borage treated with 2X “Pursuit”; The mutant borage treated with 2X “Everest 2.0”. The image was taken at 21 days after the treatment.

Table 8. Responses of the AHAS1 mutant line towards different group 2 herbicides

Commercial Name	Active ingredient	Tolerance
Accent	nicosulfuron	No
Everest 2.0	flucarbazone sodium	Yes
Express SG	tribenuron-methyl	No
Muster	ethametsulfuron methyl	No
Pinnacle SG	triflusulfuron methyl	No
PrePass XC	florasulam	No
Pursuit	imazethapyr	Yes
Solo	imazamox	Yes

4.5.6 Discussion

The herbicide dosage response test in this study provides direct evidence that the homozygous mutant line (*AHAS1*) is resistant to imidazolinones up to four times the agronomically recommended dosage. Based on visual observations, the treatment with 4X herbicide did not cause any obvious damage to the plant. In comparison with untreated control plants, the treated mutant plants showed similar growth and development (Figure 20). However, with the concentration increasing to 8X, the mutant line showed sensitivity to the herbicide. A previous study (Duggleby and Pang, 2000) showed that the mutation S653N of Arabidopsis AHAS could lead to 100 fold increase in tolerance to imidazolinones. Yet, the similar high level of resistance to the herbicide on the same mutation in borage was not observed in this study. The reason for the difference is not clear, but it may have something to do with the different genetic background of the two species. Arabidopsis possesses only one AHAS gene, while borage has two AHAS genes (*AHAS1* and *AHAS2*) to support essential AHAS activity. If one of the two

genes in borage is inhibited, the other gene might not be able to provide enough strength to tolerate a high level of the herbicide. However, the *in vitro* assays (Section 4.4) did show AHAS activity in the single gene mutant line could still maintain nearly 20% of the original activity at 625 μM of imazamox. Since the *AHAS2* mutation was also discovered from another imidazolinone resistant line, it is possible to cross the homozygous *AHAS1* mutant with the *AHAS2* mutant plant to acquire offspring containing the two mutated AHAS genes. Then, the level of herbicide resistance would be expected to increase beyond the current level.

The result of herbicide type response test has showed that the M4 imidazolinone resistant borage (*AHAS1* mutant) has an equally strong resistant level to both imazamox and imazethapyr, but zero tolerance to other herbicides except flucarbazone sodium (Figure 21). This result was different from the previous study that the S653 mutation only confers tolerance to imidazolinones, not any cross-tolerance to the other Group 2 herbicides (Dietrich, 1998; Lee *et al*., 1999; Sathasivan *et al*., 1991; Tan *et al*. 2006; Tranel and Wright, 2002; Tranel *et al.*, 2003). In contrast to imazamox and imazethapyr treatments, the wild-type control treated by flucarbazone sodium showed less injury and damage (Figure 21), indicating that the wild-type borage may naturally exhibit slight tolerance to flucarbazones by utilizing cytochrome P450 monooxygenases to convert the herbicide into non-toxic derivatives (Yuan *et al*. 2006). However, the S651N mutation of the AHAS genes in borage enhances the level of tolerance to flucarbazones. This observation has never been reported before.

5.0 GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

This thesis research aims to create, identify and characterize ethyl methanesulfonate (EMS)-induced borage mutants for herbicide imidazolinone resistance. An EMS-mutagenized population was generated using a series of concentrations of EMS to treat borage seeds. Screening of the mutant population with a group 2 herbicide resulted in identification of two stable imidazolinone-resistant lines. Molecular analysis of the mutant lines revealed a single nucleotide substitution in the *AHAS1* gene resulting in an amino acid change (S651N) in the first resistant line, and a single nucleotide substitution in the *AHAS2* gene resulting in an amino acid change (S647N) in the second resistant line. The *in vitro* assay showed the mutation in the *AHAS1* gene could retain significantly high enzymatic activity in presence of the herbicide, compared to the wild type. Based on the single nucleotide substitution in the *AHAS1* gene, the KASP marker was developed to genotype the herbicide-resistant and -susceptible plants. The herbicide resistant lines and the genotyping marker are very valuable for developing commercially viable imidazolinone-resistant borage cultivars to meet the pressing demand of borage farming.

Genome sequence analysis showed that two highly homologous AHAS genes are present in the borage genome. A single nucleotide substitution in either *AHAS1* or *AHAS2* gene resulting in an amino acid change from serine (S) to asparagine (N) could leads to herbicide resistance, indicating both *AHAS1* and *AHAS2* genes have important role for supporting growth and development. Analysis of segregation of plants in the *AHAS1* mutant line using the KASP SNP marker showed that imidazolinone resistance is dominantly inherited. *In vitro* AHAS enzymatic assay of the *AHAS1* mutant line and the herbicide dose/type response test have confirmed that the S651N mutation of the *AHAS1* is the cause for imidazolinone-resistance.

Due to the time limitation, *in vitro* assay and KASP marker development on the *AHAS2* mutant was not undertaken. However, the principle and methodology for doing that are the same provided in the Study 3 and Study 4. Characterization of the *AHAS2* mutant line would provide information on this mutant and expand the knowledge and understanding of the relationship of *AHAS1* and *AHAS2*.

Since the *AHAS2* mutation in the second imidazolinone resistant line was already identified, it is now possible to cross the homozygous AHAS1 mutant with the homozygous AHAS2 mutant to create offspring plants combining the two single mutations of both AHAS genes. The recombinant would have advantage to provide a much higher level of herbicide resistance beyond the current level with great potential for weed control in borage farming.

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7.0 APPENDICES

Appendix 1: *Borago officinalis* Wild Type (Imidazolinone Susceptible) *AHAS* Sequences

Borago officinalis Wild Type (Imidazolinone Susceptible) *AHAS1* Nucleotide Sequence

ATGGCGTCTACTCCTCCTTCCTCCACCCTCACCCACCCCACCACCACCCCCTCCTCAT
TTCCTAACCACCCAAAACTCTTCTCATCCTCCTTCACCCTTCCATTTCCTGTTTCCCCC
CAAACCACCTCCCTCTCCCACTCCAAACACCTCCGCCGACATTCCCTCCACCCAATC
TCAAACGTCATTTCCACCCGTCCTTCCACCTCATCTCCCTCTTCCCAAAATACCCCCG
AACAAAAAGAACAACTTCCATTCATTTCCAGATACGCCCCTAACGAACCAAGAAAA
GGCGCTGACGTTCTCGTTGAAGCCCTCGAAAGACAAGGAGTGACCAACGTCTTCGC
CTACCCGGGTGGCGCCTCCATGGAGATTCACCAAGCGCTTACCCGCTCCAACATTAT
TAAAAACGTGCTTCCTAGGCATGAACAGGGAGGAGTTTTTGCAGCTGAGGGATATG
CACGTGCTTCGGGCGAGCCAGGTGTTTGTATTGCTACTTCTGGACCTGGAGCGACGA
ATCTTGTTAGTGGTTTGGCTGATGCTTTGTTGGATAGTGTTCCTATGGTGGCGATTAC
TGGACAAGTACCTCGTAGGATGATTGGTACGGATGCTTTTCAAGAAACGCCTATTGT
TGAGGTAACTAGGTCGATTACCAAACATAATTATCTTGTTTTGAATGTTGATGATATT
CCTAGGATTGTTAAGGAAGCGTTTTATTTAGCAAGGAGTGGTAGGCCTGGCCCAGTT
TTGATTGATGTTCCCAAAGATATTCAGCAACAGAATGTGGTTCCTAATTGGGATGTT
GAGATGGGGTTGTGTGGTTATATTTCTAGGTTGTGTAAGCCTCCTAGTGAATTGTTGT
TGGAACAGATTGTCAGGTTGATATCTGAGGCCAAAAAGCCTGTTCTTTATGTGGGGG
GAGGGTGTTTGAATTCGAGTGAGGAGTTGAAGAGGTTTGTTGAGCTTACGGGGATTC
CTGTGGCGAGTACTTTGATGGGGTTGGGGTCTTTTCCTGGTTCAGATGAGTTGTCGTT
GCAGATGCTGGGGATGCATGGGACTGTTTATGCGAATTATGCTGTGGATAAGAGCG
ATTTGATGCTTGCATTTGGGGTTAGGTTTGATGACCGTGTGACTGGGAAGTTGGAAG
CTTTTGCTAGTAGGGCGAAGATTGTTCATATTGATATTGATCCTGCTGAGATTGGGA
AGAACAAGCAGCCTCATGTTTCGATTTGTGCAGACATTAAGCTGGCTTTAGTAGGGT
TGAATTCAATATTGGAGAAGAGAGCGGGGAATTTGAAATCAAATTTCAAGGCTTGG
AGGGAGGAGCTCAATGAACAGAAGGTGAAATATCCGTTGACGTTTAAAACGTTTGG
CGATGCTATTCCACCACAATATGCAATCCAGACTCTTGATGAATTGACTAAGGGGAA
TGCAATCATAACCACGGGTGTTGGACAACATCAGATGTGGGCTGCTCAGTTTTACAA
GTATAATCGACCGCGGCAATGGTTGACATCGGCTGGATTAGGAGCCATGGGTTTTGG
ATTGCCTGCTGCTATAGGTGCTGTGGTTGCAAGGCCTGATGCCGTTGTTGTGGATATT
GATGGTGATGGCAGCTTCCTCATGAACGTCCAGGAGTTGGCGACTATCCGTGTGGAG
AATCTCCCAGTCAAAATAATGTTGTTAAATAATCAACATTTAGGTATGGTGGTACAG
TGGGAGGATCGATTCTACAAGGCGAATAGAGCACATACATATCTTGGAGACCCAAA
TCATGAGTCCGAGATATTCCCAGACATGTTGAAGTTTGCTGACGCCTGTAATATTCC
TGCTGCTCGAGTGACAAAGAAGCATGAACTGGGAGCTGCAATTCAGAAAATGTTAG
ACACCCCCGGACCATACTTATTGGATGTCATTGTCCCACATCAAGAACATGTGTTGC
CTATGATCCCAAATGGCGGAACCTTTGATGATGTTATCGTTGAAGGTGATGGAAGAA
CTAAATACTAA

Borago officinalis Wild Type (Imidazolinone Susceptible) *AHAS2* Nucleotide Sequence

ATGACGGCTACTCCTCATTCATCCACCCTCACTCACCCCACCCCCACCCCCACCTCAT
TTCCCAGCCACCCAAAACTCTTCTCCTCCTCCTTCACCCTCCCTTTTCCCCTTTCACCC
CAAACCACCTCCCTCTCCCATACCAAACACATCCGCCGTAATTCTCTCCACCCAATC
TCAAACGTCATTTCCCCCTCTCCAATCCCCTCTTCCCAAAGTACCCCTCAACAAAAA
CAACCCCCCTTCATTTCAAGATACGCCCCTGAAGAGCCAAGAAAAGGAGCCGATGT
TCTCGTGGAAGCCTTAGAAAGAGAAGGAGTCACCAACGTCTTCGCCTACCCGGGTG
GCGCCTCTATGGAGATCCATCAGGCCCTCACCCGCTCCAACATTATTAAAAACGTGC
TTCCTAGACATGAACAGGGTGGTGTTTTCGCAGCTGAGGGATATGCACGAGCTTCGG
GCGACCCGGGTGTTTGTATTGCTACTTCTGGACCCGGTGCGACGAATCTTGTAAGTG
GGTTGGCTGATGCTTTGTTGGATAGTGTCCCTATGGTGGCGATTACTGGACAAGTTC
CTCGTAGGATGATTGGTACTGATGCGTTTCAAGAAACACCTATTGTTGAGGTAACTA
GGTCTATTACTAAACATAATTATCTTGTTTTGAGTGTTGATGATATTCCTAGGATTGT
TAAGGAAGCGTTTTATTTAGCTAGGAGTGGTAGGCCTGGCCCGGTTTTGATTGACGT
TCCTAAAGATATTCAGCAACAGATGGTGGTTCCTCATTGGGATGTTGAGATGGGGTT
GAGTGGTTATATTTCTAGGTTGTGTAAGCCGCCTTGTGAATTGTTGTTGGAACAAATT
GTGAGGTTGATTTCTGAGGCGAAAAGGCCGGTGCTTTATGTGGGAGGAGGATGTTTG
AATTCGAGTGAGGAGTTAAAGAGGTTTGTTGAGCTTACAGGGATTCCTGTGGCCAGT
ACTTTGATGGGTTTGGGGTCATTTCCTGGTTCGGATGAGTTGTCGTTGCAGATGCTGG
GGATGCATGGGACTGTTTATGCGAATTATGCTGTGGATAAGAGTGATTTGATGCTTG
CGTTTGGGGTTAGGTTTGATGATCGTGTGACTGGGAAGTTGGAAGCTTTTGCTAGTA
GGGCAAAGATTGTCCATATTGATATTGATCCTGCTGAGATTGGGAAGAACAAGCAG
CCTCATGTTTCGATTTGTGCTGACATTAAGCTGGCTTTGGCGGGGCTGAATTCGATAT
TGGAGGGGAGAGCGGGGAATTTGAAAGCAAATTTCTCGGCTTGGAGGGAGGAGCTC
AATGAACAGAAAGTGAAACATCCGTTGACATTTAAAACGTTTGGAGATGCTATTCCA
CCACAATATGCGATTCAGACTCTTGATGAATTGACTAAGGGGAATGCAATCATAAGT
ACCGGTGTTGGACAACATCAAATGTGGGCAGCTCAGTTTTACAAGTATAATCGACCA
CGGCAATGGTTGACGTCAGCTGGATTAGGAGCCATGGGATTTGGATTGCCTGCTGCT
ATAGGTGCTGTGGTTGCAAGGCCTGATGCCGTTGTTGTAGATATAGATGGTGATGGC
AGCTTCCTCATGAACGTGCAGGAGTTGGCGACTATTCGCGTGGAGAATCTCCCAGTC
AAAATCATGTTGTTAAATAATCAACATTTAGGTATGGTGGTACAGTGGGAGGACCG
ATTCTACAAGGCCAATAGAGCACATACATATCTTGGAGATCCAAATCATGAGTCCGA
GATATTCCCAGACATGTTGAAGTTTGCTGACGCCTGTAATATTCCTGCTGCTCGAGT
GACAAAGAAGAATGAACTGAGAGCTGCAATCCAGAAAATGTTAGACACCCCTGGAC
CATACTTATTGGATGTCGTTGTGCCACATCAAGAACATGTGCTGCCTATGATCCCAA
GTGGCGGAACCTTTGACGATGTTATTGTTGAAGGTGATGGAAGAACTAAATACTGA

Appendix 2: *Borago officinalis* Mutant (Imidazolinone Resistant) *AHAS1* Sequences

Borago officinalis Mutant (Imidazolinone Resistant) *AHAS1* Nucleotide Sequence

ATGGCGTCTACTCCTCCTTCCTCCACCCTCACCCACCCCACCACCACCCCCTCCTCAT
TTCCTAACCACCCAAAACTCTTCTCATCCTCCTTCACCCTTCCATTTCCTGTTTCCCCC
CAAACCACCTCCCTCTCCCACTCCAAACACCTCCGCCGACATTCCCTCCACCCAATC
TCAAACGTCATTTCCACCCGTCCTTCCACCTCATCTCCCTCTTCCCAAAATACCCCCG
AACAAAAAGAACAACTTCCATTCATTTCCAGATACGCCCCTAACGAACCAAGAAAA
GGCGCTGACGTTCTCGTTGAAGCCCTCGAAAGACAAGGAGTGACCAACGTCTTCGC
CTACCCGGGTGGCGCCTCCATGGAGATTCACCAAGCGCTTACCCGCTCCAACATTAT
TAAAAACGTGCTTCCTAGGCATGAACAGGGAGGAGTTTTTGCAGCTGAGGGATATG
CACGTGCTTCGGGCGAGCCAGGTGTTTGTATTGCTACTTCTGGACCTGGAGCGACGA
ATCTTGTTAGTGGTTTGGCTGATGCTTTGTTGGATAGTGTTCCTATGGTGGCGATTAC
TGGACAAGTACCTCGTAGGATGATTGGTACGGATGCTTTTCAAGAAACGCCTATTGT
TGAGGTAACTAGGTCGATTACCAAACATAATTATCTTGTTTTGAATGTTGATGATATT
CCTAGGATTGTTAAGGAAGCGTTTTATTTAGCAAGGAGTGGTAGGCCTGGCCCAGTT
TTGATTGATGTTCCCAAAGATATTCAGCAACAGAATGTGGTTCCTAATTGGGATGTT
GAGATGGGGTTGTGTGGTTATATTTCTAGGTTGTGTAAGCCTCCTAGTGAATTGTTGT
TGGAACAGATTGTCAGGTTGATATCTGAGGCCAAAAAGCCTGTTCTTTATGTGGGGG
GAGGGTGTTTGAATTCGAGTGAGGAGTTGAAGAGGTTTGTTGAGCTTACGGGGATTC
CTGTGGCGAGTACTTTGATGGGGTTGGGGTCTTTTCCTGGTTCAGATGAGTTGTCGTT
GCAGATGCTGGGGATGCATGGGACTGTTTATGCGAATTATGCTGTGGATAAGAGCG
ATTTGATGCTTGCATTTGGGGTTAGGTTTGATGACCGTGTGACTGGGAAGTTGGAAG
CTTTTGCTAGTAGGGCGAAGATTGTTCATATTGATATTGATCCTGCTGAGATTGGGA
AGAACAAGCAGCCTCATGTTTCGATTTGTGCAGACATTAAGCTGGCTTTAGTAGGGT
TGAATTCAATATTGGAGAAGAGAGCGGGGAATTTGAAATCAAATTTCAAGGCTTGG
AGGGAGGAGCTCAATGAACAGAAGGTGAAATATCCGTTGACGTTTAAAACGTTTGG
CGATGCTATTCCACCACAATATGCAATCCAGACTCTTGATGAATTGACTAAGGGGAA
TGCAATCATAACCACGGGTGTTGGACAACATCAGATGTGGGCTGCTCAGTTTTACAA
GTATAATCGACCGCGGCAATGGTTGACATCGGCTGGATTAGGAGCCATGGGTTTTGG
ATTGCCTGCTGCTATAGGTGCTGTGGTTGCAAGGCCTGATGCCGTTGTTGTGGATATT
GATGGTGATGGCAGCTTCCTCATGAACGTCCAGGAGTTGGCGACTATCCGTGTGGAG
AATCTCCCAGTCAAAATAATGTTGTTAAATAATCAACATTTAGGTATGGTGGTACAG
TGGGAGGATCGATTCTACAAGGCGAATAGAGCACATACATATCTTGGAGACCCAAA
TCATGAGTCCGAGATATTCCCAGACATGTTGAAGTTTGCTGACGCCTGTAATATTCC
TGCTGCTCGAGTGACAAAGAAGCATGAACTGGGAGCTGCAATTCAGAAAATGTTAG
ACACCCCCGGACCATACTTATTGGATGTCATTGTCCCACATCAAGAACATGTGTTGC
CTATGATCCCAAGTGGCGGAACCTTTGATGATGTTATCGTTGAAGGTGATGGAAGAA
CTAAATACTAA

Borago officinalis Mutant (Imidazolinone Resistant) *AHAS2* Nucleotide Sequence

ATGACGGCTACTCCTCATTCATCCACCCTCACTCACCCCACCCCCACCCCCACCTCAT
TTCCCAGCCACCCAAAACTCTTCTCCTCCTCCTTCACCCTCCCTTTTCCCCTTTCACCC
CAAACCACCTCCCTCTCCCATACCAAACACATCCGCCGTAATTCTCTCCACCCAATC
TCAAACGTCATTTCCCCCTCTCCAATCCCCTCTTCCCAAAGTACCCCTCAACAAAAA
CAACCCCCCTTCATTTCAAGATACGCCCCTGAAGAGCCAAGAAAAGGAGCCGATGT
TCTCGTGGAAGCCTTAGAAAGAGAAGGAGTCACCAACGTCTTCGCCTACCCGGGTG
GCGCCTCTATGGAGATCCATCAGGCCCTCACCCGCTCCAACATTATTAAAAACGTGC
TTCCTAGACATGAACAGGGTGGTGTTTTCGCAGCTGAGGGATATGCACGAGCTTCGG
GCGACCCGGGTGTTTGTATTGCTACTTCTGGACCCGGTGCGACGAATCTTGTAAGTG
GGTTGGCTGATGCTTTGTTGGATAGTGTCCCTATGGTGGCGATTACTGGACAAGTTC
CTCGTAGGATGATTGGTACTGATGCGTTTCAAGAAACACCTATTGTTGAGGTAACTA
GGTCTATTACTAAACATAATTATCTTGTTTTGAGTGTTGATGATATTCCTAGGATTGT
TAAGGAAGCGTTTTATTTAGCTAGGAGTGGTAGGCCTGGCCCGGTTTTGATTGACGT
TCCTAAAGATATTCAGCAACAGATGGTGGTTCCTCATTGGGATGTTGAGATGGGGTT
GAGTGGTTATATTTCTAGGTTGTGTAAGCCGCCTTGTGAATTGTTGTTGGAACAAATT
GTGAGGTTGATTTCTGAGGCGAAAAGGCCGGTGCTTTATGTGGGAGGAGGATGTTTG
AATTCGAGTGAGGAGTTAAAGAGGTTTGTTGAGCTTACAGGGATTCCTGTGGCCAGT
ACTTTGATGGGTTTGGGGTCATTTCCTGGTTCGGATGAGTTGTCGTTGCAGATGCTGG
GGATGCATGGGACTGTTTATGCGAATTATGCTGTGGATAAGAGTGATTTGATGCTTG
CGTTTGGGGTTAGGTTTGATGATCGTGTGACTGGGAAGTTGGAAGCTTTTGCTAGTA
GGGCAAAGATTGTCCATATTGATATTGATCCTGCTGAGATTGGGAAGAACAAGCAG
CCTCATGTTTCGATTTGTGCTGACATTAAGCTGGCTTTGGCGGGGCTGAATTCGATAT
TGGAGGGGAGAGCGGGGAATTTGAAAGCAAATTTCTCGGCTTGGAGGGAGGAGCTC
AATGAACAGAAAGTGAAACATCCGTTGACATTTAAAACGTTTGGAGATGCTATTCCA
CCACAATATGCGATTCAGACTCTTGATGAATTGACTAAGGGGAATGCAATCATAAGT
ACCGGTGTTGGACAACATCAAATGTGGGCAGCTCAGTTTTACAAGTATAATCGACCA
CGGCAATGGTTGACGTCAGCTGGATTAGGAGCCATGGGATTTGGATTGCCTGCTGCT
ATAGGTGCTGTGGTTGCAAGGCCTGATGCCGTTGTTGTAGATATAGATGGTGATGGC
AGCTTCCTCATGAACGTGCAGGAGTTGGCGACTATTCGCGTGGAGAATCTCCCAGTC
AAAATCATGTTGTTAAATAATCAACATTTAGGTATGGTGGTACAGTGGGAGGACCG
ATTCTACAAGGCCAATAGAGCACATACATATCTTGGAGATCCAAATCATGAGTCCGA
GATATTCCCAGACATGTTGAAGTTTGCTGACGCCTGTAATATTCCTGCTGCTCGAGT
GACAAAGAAGAATGAACTGAGAGCTGCAATCCAGAAAATGTTAGACACCCCTGGAC
CATACTTATTGGATGTCGTTGTGCCACATCAAGAACATGTGCTGCCTATGATCCCAA
ATGGCGGAACCTTTGACGATGTTATTGTTGAAGGTGATGGAAGAACTAAATACTGA

Appendix 3 Statistical Analysis of AHAS Enzyme Activity

Effects of borage genotype and herbicide concentrations on AHAS enzyme activity from *in vitro* assay.

Borage genotype	Herbicide concentration (uM)	AHAS activity (%)
Resistant	0	100a
	1	81b
	5	62c
	25	39e
	125	32e
	625	15f
Susceptible	0	100a
	1	68c
	5	51d
	25	14f
	125	5fg
	625	2g
SEM	2.071	
Statstical Analysis	P value	
Genotype effect (G)	< .0001	
Herbicide concentration effect (H)	< .0001	
G * H interaction effect	< .0001	

Note: Means with the same letter in the same column are not significantly different (P >0.05). The multi-treatment comparisons is using Tukey method. SEM = stantard error of mean. Genotypes, herbicide concentrations and the interaction of genotype variety*herbicide concentrations all showed significant effects on AHAS enzyme activity because their P values are less than 0.05.